Population Genetic Structure of the Ground Pangolin based on Mitochondrial Genomes

Zelda du Toit

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POPULATION GENETIC STRUCTURE OF THE GROUND PANGOLIN BASED ON MITOCHONDRIAL GENOMES

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This study is dedicated to both my loving grandfather Gert Johannes van Rhyn
and mother Marlene Burbidge for their unconditional love, support
and understanding as well as for motivating me to follow my dreams.

DECLARATION AND COPYRIGHT

I declare that the dissertation hereby submitted by me for the *Magister Scientiae* degree at the University of the Free State is my own independent work and has not previously been submitted by me at another University/Faculty. I furthermore concede copyright of the dissertation in favour of the University of the Free State.

Zelda du Toit
31 March 2014

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All samples were obtained by qualified individuals or veterinarians to ensure as little harm as possible were done to the animals. This study was submitted to the NZG’s research and ethics committee, where ethics and legislative compliance were evaluated and approved. The NSPCA also played a role in the evaluation process at the NZG. TOPS permits in addition to Provincial collection permits to transport tissue between provinces were obtained for sampling. No individual pangolins were harmed or adversely affected as a direct result of this study.

TABLE OF CONTENTS

PAGE NUMBER

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

CHAPTER TWO

ISOLATION AND CHARACTERIZATION OF MITOCHONDRIAL DNA MARKERS FOR THE TEMMINCK’S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*)

CHAPTER THREE

SCALE SAMPLES FROM TEMMINCK’S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*); A NON-INVASIVE SOURCE OF DNA

CHAPTER FOUR

THE COMPLETE MITOCHONDRIAL GENOME OF TEMMINCK’S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*; SMUTS, 1932) AND PHYLOGENETIC POSITION OF PHOLIDOTA (WEBER, 1904)

CHAPTER FIVE

MOLECULAR PHYLOGEOGRAPHY OF TEMMINCK’S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*) BASED ON MITOCHONDRIAL DNA VARIATION

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

LIST OF ABBREVIATIONS

A260/280	Ratio of absorptions at 260 nm vs 280 nm
AIC	Akaike Information Criterion
AMOVA	Analysis of Molecular Variance
APWG	African Pangolin Working Group
ATP	Adenosine Triphosphate
BI	Bayesian Inference
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
bp	Basis pare
BSA	Bovine Serum Albumin
C	Conserved Sites
COI	Cytochrome *c* oxidase I
Cyt*b*	Cytochrome *b*
ddH_2O	Double Distilled Water
dNTP	Deoxyribonucleotide Triphosphate
DTT	Dichlorodiphenyltrichloroethane
EDTA	Ethylene Diamine Tetra-acetic Acid
ESU	Evolutionary Significant Units
F_{CT}	Fixation Index (Among Groups)
Fsc	Among Populations Within Groups
F_{ST}	Fixation Index (Genetic Differentiation)
G	Gamma Parameter
G_{ST}	Genetic Distance
H_T	Haplotype Diversity
I	Invariant Sites
IUCN	International Union for the Conservation of Nature
kya	Thousand Years Ago
M	Molar

$MgCl_2$	Magnesium Chloride
ML	Maximum Likelihood
mya	Million Years Ago
n	Nano (10^{-9})
ng	Nanograms
NCBI	National Centre for Biotechnology Information
NJ	Neighbor-Joining
NRF	National Research Foundation
NZG	National Zoological Gardens of South Africa
Pi	Parsimony Informative
PNG	Pangolin
R	Transition:Transversion Ratio
STR	Short Tandem Repeats
SSR	Simple Sequence Repeats
SNPs	Single Nucleotide Polymorphisms
S*ry*	Sex-determining Region
ssDNA	Single Stranded DNA
V	Variable Sites
VHF	Very High Frequency

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. BACKGROUND INFORMATION

Temminck's ground pangolin (*Smutsia temminckii*) is the only pangolin species that occurs within southern Africa. This species has the largest distribution range of all four African pangolins and their range extends from South Africa northwards and eastwards in Africa as far as Chad (Heath, 1992; Skinner & Chimimba, 2005; Soewu & Ayodele, 2009). Temminck's ground pangolin belongs to the order Pholidota which consists of three genera. Of the eight global pangolin species, the four species that occur in Asia fall under the genus *Manis* and the four African species are divided into two groups: two arboreal species under the genus *Phataginus* and the two ground-dwelling species under *Smutsia*.

Pangolins play an important role in the ecosystem since they feed on termites and ants, thus controlling the numbers of these invertebrates. An adult pangolin can consume approximately 70 million insects per year (Chao, 2002). In China, pangolins perform a significant role in limiting termites from human dwellings and are listed as a second-class protected animal (Heath, 1992; Whitfort, 2012). Very little research has been undertaken on Africa's pangolins and even though Temminck's ground pangolin is the most widely distributed, very limited information is available on the ecology and behaviour of this species (Heath, 1992; Akpona *et al*., 2008). However, this species is under threat from poaching primarily for traditional medicine (Bräutigam *et al*., 1994) as well as electrocution by game fences, especially in southern Africa (Pietersen *et al*., 2014*a*). The bush-meat trade and *Muthi* markets is a significant cause of mortality within all African pangolin species (Sodeinde & Adedipe, 1994; Soewu, 2008; Soewu & Adekanola, 2011) and, in recent years, there have been significant increases in the export of African pangolins to Asia. In Asia, pangolins are regarded as a delicacy and are used in traditional medicinal practices (Newton *et al*., 2008; Challender & Hywood, 2012).

Currently, no genetic studies have been carried out on Temminck’s ground pangolins and therefore no information is available that can be sourced to determine population genetic structure across their distribution range. This study investigated the phylogenetics of sub-populations of Temminck’s ground pangolin, *S. temminckii*, in southern Africa using full mitochondrial deoxyribonucleic acid (mtDNA) sequencing. The results aimed to present the amount of genetic variation within southern Africa and determine whether they should be managed as sub-populations. The latter will be necessitated if molecular variation between populations is of significant magnitude to warrant consideration as Evolutionary Significant Units (ESUs).

A comparison of Temminck’s ground pangolin with other Asian and African pangolins elucidated their divergent evolutionary traits (Luo *et al*., 2007) and allowed us to use genetic markers to determine genetic structure and genetic relationships between species. In turn, this, along with future research, will create a framework for better conservation and management plans as well as captive breeding and relocation programs where necessary (Crozier, 1997).

This study complemented the recent international research program launched towards the better conservation of all pangolin species that are currently listed as threatened. The program is supported by the International Union for the Conservation of Nature’s Pangolin Specialist Group and the South African pangolin conservation initiative – the African Pangolin Working Group.

1.2. PANGOLIN OVERVIEW

1.2.1. TAXONOMY OF PANGOLINS

The pangolin is a mammal belonging to the order Pholidota (Weber, 1904; Nisa *et al.*, 2005; Ntiamoa-Baidu *et al*., 2005) and the family Manidae (Gray, 1821). There are eight species of pangolins in the world (Figure 1.1) of which four are found in Asia and the other four in Africa (Herklots, 1937; Gaudin & Wible, 1999; Gaubert & Antunes, 2005; Skinner & Chimimba, 2005; Wilson & Reeder, 2005; Soewu & Ayodele, 2009; Hsieh *et al.*, 2011). The Asian species differ from the African ones primarily by having hairs that are layered between the scales, whereas the African species do not (Herklots, 1937; Dickman & Richer, 2001). The Asian species include the Philippine pangolin, *Manis culionensis* (De Elera, 1895), Indian or thick-tailed pangolin, *M. crassicaudata* (Geoffroy, 1803), Chinese pangolin, *M. pentadactyla* (Linnaeus, 1758) and Malayan/Sunda pangolin, *M. javanica* (Desmarest, 1822). All pangolins previously belonged to the genus *Manis* (Linnaeus, 1758), but the two African ground pangolin species were later reclassified into the genus *Smutsia* (Gray, 1865) and the two African tree pangolin species to the genus *Phataginus* (Rafinesque, 1821). However, some authors still refer to all the African species as *Manis* (Gaudin & Wible, 1999). The four African species include the two arboreal species, namely the tree pangolin (also known as the African white-bellied pangolin or three-cusped pangolin), *Phataginus tricuspis*, (Rafinesque, 1821) and the long-tailed pangolin (or black-bellied pangolin), *P. tetradactyla* (Linnaeus, 1766). The two ground-dwelling species include the giant ground pangolin, *Smutsia gigantea* (Illiger, 1815) and Temminck's ground pangolin (or Cape pangolin), *S. temminckii* (Smuts, 1832).

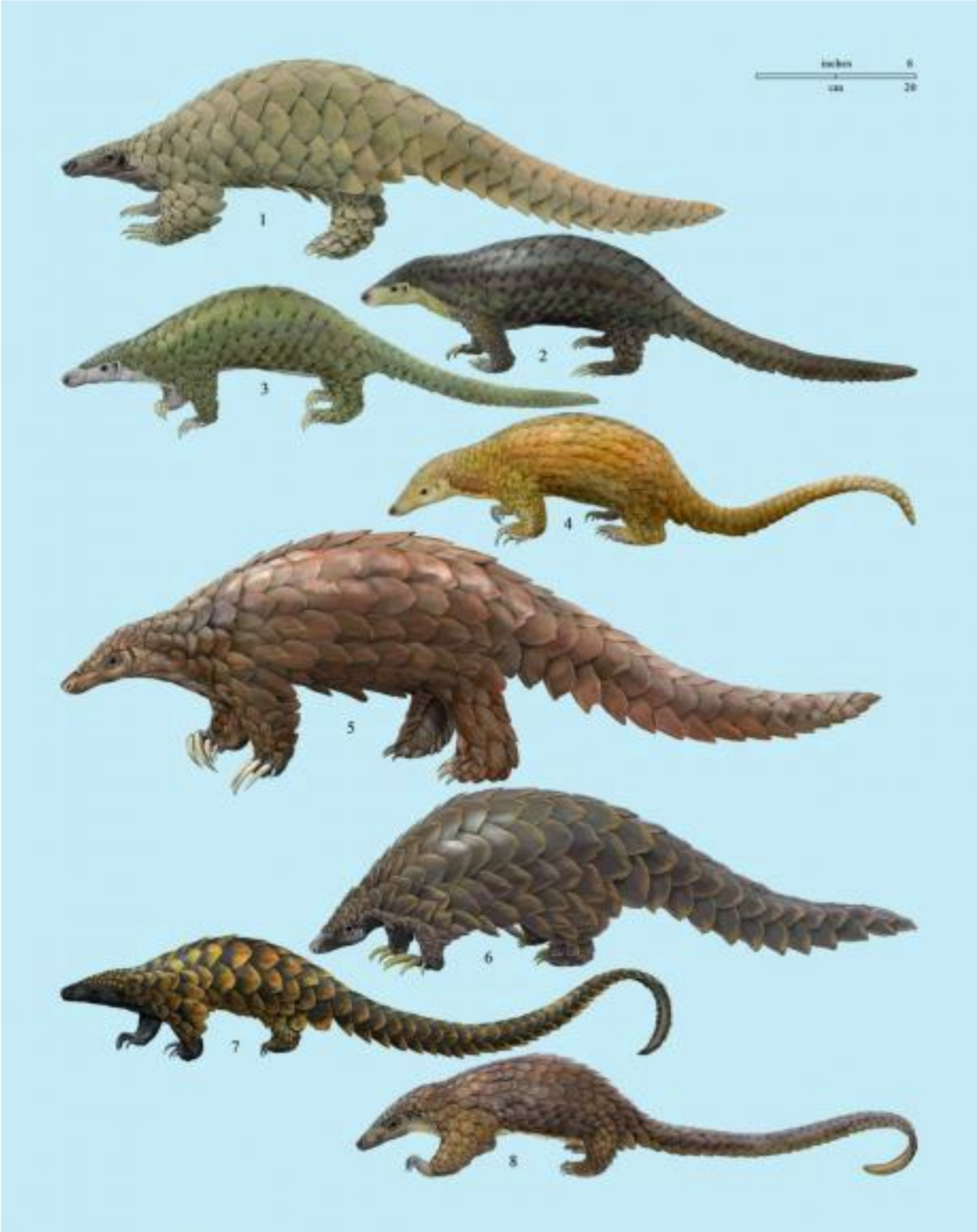

Figure 1.1: Illustration of all eight extant species of pangolins. (1) Indian or thick-tailed pangolin, *Manis crassicaudata;* (2) Chinese pangolin, *M. pentadactyla;* (3) Malayan or Sunda pangolin, *M. javanica;* (4) Philippine pangolin, *M. culionensis;* (5) Giant ground pangolin, *Smutsia gigantea*; (6) Temminck's ground pangolin or Cape pangolin, *S. temminckii*; (7) The long-tailed pangolin or black-bellied pangolin, *Phataginus tetradactyla*; (8) African white-bellied pangolin or three-cusped pangolin, *P. tricuspis* (Illustration: Llobit, 2011).

1.2.2. THREATS TO PANGOLINS

1.2.2.1. HUMAN-PANGOLIN CONFLICT

According to Beck (2008), Komen (2009) and Pietersen *et al.* (2014*a*), electrocution on electric fences (Figure 1.2a) constitutes the most significant threat to pangolins in southern Africa. These electrified fences are established mostly around private nature reserves and game farms to either keep animals in or prevent problem animals from entering. Electrocutions occur when a pangolin approach the lower electrified wire of a fence in an upright position. Once shocked; it instinctively curls up into a ball, attempting to make use of its natural method of defence to protect itself from harm. However, repeated electric pulses eventually cause death (Beck, 2008; Komen, 2009; Pietersen *et al.*, 2014*a*).

1.2.2.2. HABITAT LOSS AND CLIMATE CHANGE

The pangolin's natural habitat has shown a gradual decline in all habitats worldwide and has been destroyed over time due to human actions and development (Sodeinde & Adedipe, 1994; Yang *et al.*, 2007; Whiting *et al.*, 2011). This has led to a decrease in the pangolins' food availability which in turn has led to a population decline in all eight pangolin species (Sodeinde & Adedipe, 1994; Yang *et al.*, 2007). Habitat loss was most likely responsible for the major decline in pangolin numbers in the Kwa-Zulu Natal Province, South Africa (Friedman & Daly, 2004).

Figure 1.2: (a) Temminck's ground pangolin caught on an electric fence (Illustration: Pietersen, 2013); (b) Pangolin scales confiscated from poachers in Malaysia (Illustration: Lumpur, 2009); (c) Pangolin foetus soup: pangolins are targeted as a food source in Africa and a delicacy in Asia (Illustration: Anderson, 2009).

1.2.2.3. ILLEGAL HUNTING AND TRADITIONAL MEDICINE

Over-exploitation has contributed vastly to the decline of various species' numbers globally and the pangolin is no exception. These animals are widely sought after by both the Asian and African markets for various reasons. The majority of animals harvested are for traditional medicine purposes and as a food source.

MUTHI MARKET: One of the biggest threats in Africa is the over-exploitation of wildlife and endangered species due to illegal hunting and the increased demand for traditional medicine (Bräutigam *et al.*, 1994; Sodeinde & Adedipe, 1994; Alves & Rosa, 2005; Soewu & Ayodele, 2009; Chakravorty *et al.*, 2011). The pangolin and particularly Temminck's ground pangolin is considered to be one of the most commonly used animals in traditional medicine (Soewu & Ayodele, 2009). Temminck's ground pangolin meat, body parts and scales (Figure 1.2b) are sold and used to create traditional medicines in various parts of Africa (Sodeinde & Adedipe, 1994; Soewu, 2008; Soewu & Ayodele, 2009; Soewu & Adekanola, 2011). According to Sodeinde & Adedipe (1994); Soewu (2006); Semiadi *et al.* (2009); Soewu & Ayodele (2009) and Chakravorty *et al.* (2011) the increase in demand for medicinal animals in order to produce traditional medicine will have a major impact on biodiversity and eventually lead to a great decline and later extinction in certain species, especially Temminck's ground pangolin. The scales appear to be the most widely used part of the animal for medical or traditional uses (Soewu & Adekanola, 2011). The value of Pangolin products are very high and can vary anything from \$600–\$650 per kilogram of scales on the *Muthi* markets (Andersson, 2014; Davies, 2014). In Zimbabwe prices range between \$7,000 and \$10,000 per pangolin (Hywood, 2013) and in Namibia it starts at \$1500 per individual (Nebe & Rankin, 2013).

ASIAN MARKET: Despite serving as a form of pest control by preying on termites, the Chinese pangolin continues to be severely exploited in numerous ways and achieved the status of *Critically Endangered* on the IUCN Red Data List (Heath, 1992; Whitfort, 2012; IUCN, 2014). This has led to an increase in use of pangolin meat and scales, sources within Asia (Figure 1.2b,c) or imported from Africa (Newton *et al*., 2008; Pantel & Chin, 2009; Pantel & Anak, 2010; Challender & Hywood, 2012). Trade and export of pangolins and pangolin products, especially the scales, is of great concern due to the high demand for scales for medicinal use. The scales are usually ground into a powder and consumed to increase blood circulation as well as fight various diseases (Fang & Wang, 1980). According to Harrisson & Loh (1965), pangolins have been targeted for decades and it is estimated that more than 60 tons of scales from pangolins were legally exported from Borneo, Malaysia in a six year period ranging from 1958 to 1964.

1.3. TEMMINCK'S GROUND PANGOLIN (*S. TEMMINCKII*)

1.3.1. GEOGRAPHICAL DISTRIBUTION OF THE SPECIES

Heath (1992) and Skinner & Chimimba (2005) stated that Temminck's ground pangolin has the largest distribution range (Figure 1.3) of all four African species and is also the only pangolin occurring in the southern African region. The range of this species extends from the northern parts of South Africa (Northern Cape, Northwest Province, Limpopo Province, Mpumalanga, Free State and Kwa-Zulu Natal) through Namibia, Zimbabwe and Botswana to northeast Africa (Ethiopia, Malawi, Zambia and Chad) (Heath, 1992; Skinner & Chimimba, 2005; Soewu & Ayodele, 2009). They are found in semi-arid habitats such as savannahs and grasslands and occur rarely in woods, forest and swamps since they prefer habitats with drier climates and lower rainfall (Skinner & Chimimba, 2005). However, they do sometimes occur in habitats such as bushveld, savannah woodlands, floodplains and rocky areas where higher rainfall occurs (Skinner & Chimimba, 2005; Soewu & Ayodele, 2009).

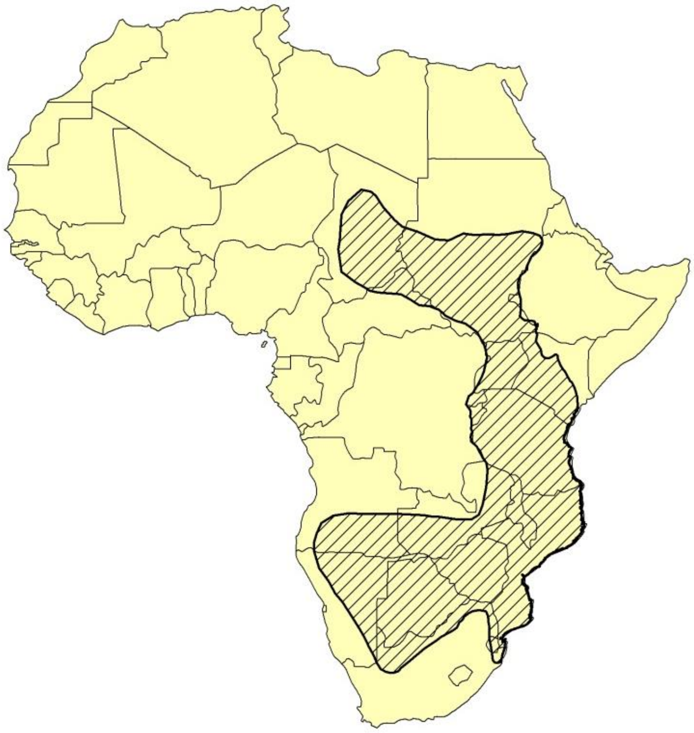

Figure 1.3: Distribution map of Temminck's ground pangolin (*S. temminckii*) (Illustration: Pietersen *et al.*, 2014*b*).

1.3.2. BEHAVIOUR OF THE SPECIES

According to Herklots (1937); Robinson (1983); Heath & Vanderlip (1988); Skinner & Smithers (1990); Heath (1992, 1995); Heath & Coulson (1998); Dickman & Richer (2001); Coggins (2004); Skinner & Chimimba, (2005); Wilson & Reeder (2005); Luo *et al.* (2007); Davit-Béal *et al.* (2009); Soewu & Ayodele (2009) and Dollens (2010) the pangolin is a unique mammal since its entire body is covered in sharp overlapping scales. When threatened these animals roll into a ball, keeping the softer, most vulnerable parts on the inside and the sharp overlapping scales on the outside. This is a very effective defence mechanism since they keep swinging the tail from side to side and the sharp scales can inflict injuries to a predator (Heath, 1992). Despite this effective defence, Temminck's ground pangolin is preyed on by a number of predators such as lions (Herklots, 1937; Wilson & Reeder, 2005; Soewu & Ayodele, 2009); leopards, hyenas and pythons (Soewu & Ayodele, 2009) who manage to penetrate their defence. These animals are solitary predominantly nocturnal animals (Herklots, 1937; Sweeney, 1956; Fang & Wang, 1980; Heath & Vanderlip, 1988; Heath & Coulson, 1997*a*; Skinner & Chimimba, 2005; Tong *et al.*, 2007) and live in burrows made by other species (Herklots, 1937; Heath & Coulson, 1997*a*; Coggins, 2004; Skinner & Chimimba, 2005; Tong *et al.*, 2007). This behaviour makes them difficult to observe and study during the day. They predominantly walk upright using their tail and can cover long distances in a short period of time (Herklots, 1937; Dickman & Richer, 2001). The surface of the pangolin scales is coarse and has abrasive uneven grooves that get formed over time (Tong *et al.*, 2007). As they dig holes in the ground and enter their burrows, the scales gets grated against the soil and rocks which leads to the formation of the grooves over time. As the pangolin gets older, the uneven scales become even harder and more defined (Tong *et al.*, 2007). The remains of deceased pangolin are seldom observed, since they go underground when they are ill, hurt or starving and die within their burrows (Heath & Coulson, 1997*a*).

According to a study performed by Jacobsen *et al*. (1991) and Heath & Coulson (1997*a,b*) pangolins, and in particular Temminck's ground pangolin, have specific home ranges that they inhabit for a few years at a time. The size of the home range as well as the amount of burrows depends on the size of the pangolin (Jacobsen *et al*., 1991; Heath & Coulson, 1997*a,b*). Jacobsen *et al.* (1991) conducted a study where they released confiscated pangolins, found at a *Muthi* market, back into the wild. The animals that were released close to their home ranges migrated back to these areas whereas the ones that were relocated to another location were found dead after a few days. The precise cause of death was unknown, however it appeared that they were victims of predation or hunting, but it could also have been a result of stress due to the long distance travelled or fatigue. This habitat specificity makes rehabilitating and relocating pangolins back into the wild problematic (Jacobsen *et al.*, 1991; Wilson, 1994; Heath & Coulson, 1997*a,b*).

1.3.3. FEEDING ECOLOGY OF THE SPECIES

Pangolins are entirely myrmecophagous in that they feed solely on ants and termites. They have strong lower front limbs with extensive claws to access termites' nests and cavities (Phillips, 1926; Herklots, 1937; Gebo & Rasmussen, 1985; Skinner & Smithers, 1990; Heath, 1992; Dickman & Richer, 2001; Skinner & Chimimba, 2005; Lim & Ng, 2007; Yang *et al.*, 2007; Davit-Béal *et al.*, 2009; Soewu & Ayodele, 2009). The giant ground pangolin is known to dig deep into termite nests in order to access termites whereas Temminck's ground pangolin mostly forage near the surface since they are smaller in size and are not regarded as efficient diggers (Swart *et al.*, 1999). Pangolins are primarily nocturnal and mostly feed at night (Pagés, 1975; Heath, 1992; Richer *et al.*, 1997; Swart, 1997; Dickman & Richer, 2001) but are known to forage in the late afternoon during winter in arid areas (Pietersen, 2013). Due to their specialized feeding requirements, these animals are notoriously difficult to keep in captivity as their diet of ants and termites cannot be maintained within a captive environment and the substituted food formula does not fulfil their dietary needs (Heath, 1992; Yang *et al.*, 2007). Pangolins have very small toothless jaws (Robinson, 1983; Gebo & Rasmussen, 1985; Skinner & Smithers, 1990; Dickman & Richer, 2001; Scally *et al.*, 2001; Luo *et al.*, 2007; Ofusori *et al.*, 2007; Davit-Béal *et al.*, 2009) and a long, sticky and very muscular tongue to reach and bind to termites and ants within their nests (Doran & Allbrook, 1973; Gebo & Rasmussen, 1985; Skinner & Smithers, 1990; Heath, 1992; Chan, 1995; Dickman & Richer, 2001; Luo *et al.*, 2007; Davit-Béal *et al.*, 2009). The tongue extends all the way from the abdominal cavity (Doran & Allbrook, 1973; Heath, 1992; Chan, 1995) and can range anything from 30 cm to 75 cm, depending on the species (Doran & Allbrook, 1973). Pangolin eyesight is very limited and therefore these animals rely on their sense of smell to locate termite nests and termites that are on the ground or in trees (Dickman & Richer, 2001).

Figure 1.4: (a) Temminck's ground pangolin with baby characteristically riding on the mother's back (Illustration: Diekmann, 2012); (b) Temminck's ground pangolin swimming (Illustration: Wright, 2013).

Pangolins only have one offspring per breeding season (Figure 1.4a) which is carried on the back of the mother's tail (Phillips, 1926; Van Ee, 1966, Pagés, 1975; Van Ee, 1978; Fang & Wang, 1980; Jacobsen *et al*., 1991, Dickman & Richer, 2001; Soewu & Adekanola, 2011). It is very rare for pangolins to give birth to twins, but Jacobsen *et al*. (1991) reported witnessing this on one occasion. When the pangolin is in danger, the young animal will roll up underneath its mother and she will roll up over him to protect him. However, when the young pangolin gets older only its head get tucked in underneath its mother and its tail wraps around her. She covers him as much as possible, and in the end both of their heads are covered which acts as a double barrier against predators (Phillips, 1926; Dickman & Richer, 2001; Skinner & Chimimba, 2005).

Pangolins are reported to be excellent swimmers (Figure 1.4b) (Fang, 1981; Lewis, 1991; Dickman & Richer, 2001; Skinner & Chimimba, 2005; Yang *et al*., 2007) and are capable of swimming long distances (Yang *et al.*, 2007). It has also been reported that pangolins enjoy spending time in water masses e.g. in zoos where zookeepers observed this behaviour while cleaning the enclosures (Yang *et al.*, 2007).

1.3.4. CONSERVATION STATUS OF THE SPECIES

Temminck's ground pangolins' status is currently listed as *Vulnerable* by the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species (Pietersen *et al*., 2014*b*). Although the precise number of individuals remaining is unclear, their numbers appear to be rapidly decreasing. It is believed to already be extinct in most parts of Kwa-Zulu Natal and the Free State Province of South Africa (Friedman & Daly, 2004; Pietersen *et al*., 2014*b*). Swart *et al.* (1999) suggests that a possible cause for the extinction in these provinces might be the change in behaviour of their primary food source (*Anoplolepis custodiens*), due to climate and temperature change. Temminck's ground pangolin is listed in Zimbabwe as a specially protected animal according to the Parks and Wildlife Act 20:14, Schedule Six 123/1991 (Connelly, 2013) and is considered to be one of the top ten protected utilized species (Griffin, 1998). There are various factors that contribute to the decrease in population size and one of these factors is over-exploitation, which is greatly compounded by the fact that vast numbers of pangolins are smuggled out to southeast Asia on a yearly basis. Further detrimental factors include the loss of suitable habitats, the low breeding rate of pangolins, indiscriminate application of pesticides (Swart, 1997) and electrocution on farms (Swart, 1997; Komen, 2009). Of these factors, the biggest threats facing the Temminck's ground pangolin in southern Africa at the moment is poaching for illegal trade and electrocution on game fencing.

1.4. PANGOLIN GENETICS

1.4.1. CONSERVATION AND GENETICS

Conserving nature and biodiversity has presented a challenge for researchers in recent times since it is estimated that more than 3000 species go extinct annually (Woodruff, 2001) and around 50% of all animal species are considered either *Critically Endangered, Endangered* or *Vulnerable* to extinction (Baillie *et al.*, 2004). This is mainly due to over-exploitation of species, destruction of habitat for agriculture and logging, as well as the introduction of invasive species (Hoffmann *et al.*, 2010). Genetics can be considered an important tool in conservation since it can contribute to a better understanding of populations by determining the evolutionary linages of species, as well as to help describe population and ecological structure within and between populations (Allendorf *et al.*, 2013). Conservation and genetics have been used in combination and can assist in establishing a more accurate understanding of the structure and dynamics of natural populations. This, in turn, can contribute to better conservation and distribution strategies that included insight from an evolutionary perspective (Frankel, 1974).

Conservation genetics can be divided into five main categories (Allendorf *et al.*, 2013):

- Managing captive populations and reintroducing them into the wild as well as restoring natural populations.
- Identifying and describing individuals, determining population structure as well as kin and taxonomic relationships in groups.
- Measuring and estimating what the effect of habitat loss, habitat fragmentation and isolation of populations will be.
- Measuring and estimating the effect of hybridization and introgression within populations.
- Understanding adaptation, genetic fitness and genetic characteristics of populations and individuals.

An important aspect of conservation genetics is to measure the influence of landscape features and environmental variables on dispersal, gene flow and genetic variation of populations (Manel *et al*., 2003; Holderegger & Wagner, 2008). Such studies on phylogeography could contribute to improvement of the accuracy and precision of identification of boundaries for populations (Guillot *et al*., 2005; Palsbøll *et al*., 2007). Phylogeography can also assist in establishing phylogenetic patterns between different species and populations (Avise, 2009) that has been geographically isolated for an extended amount of time (Allendorf *et al.*, 2013). This is mostly expected to occur in populations with limited mobility or among populations separated by barriers (rivers, mountains, deserts, forest, roads, human barriers etc.).

Molecular genetics can assist in identifying the phylogenetic and taxonomic relationships among species or populations, which is an important factor in order to design effective biodiversity strategies and conservation plans. In early years, phenotype was the only way to distinguish between different species and this was the dominant system for many years. However, genetics have been implemented to establish taxonomy using genotypic characteristics. As a result, phylogenetics is used to establish similar genetic traits shared from a common ancestor between different organisms in order to more accurately define classifications of species (Allendorf *et al.*, 2013).

1.4.2. DEVELOPMENT OF GENETIC MAKERS

Deoxyribonucleic acid (DNA) can be observed in both the nucleus and mitochondrion of a cell and often codes for specific proteins (Castro *et al.*, 1998). There are a vast variety of different types of markers available depending on the specific application. These markers can include both cross-species and species-specific markers obtained for nuclear DNA studies, Y-chromosome studies, developing Single Nucleotide Polymorphisms (SNPs) and for mtDNA.

1.4.2.1. MICROSATELLITE MARKERS

Microsatellites are also referred to as short tandem repeats (STRs) or simple sequence repeats (SSRs). These regions are considered the most variable type of sequence in the genome and are characterized by high heterozygosity and multiple alleles which creates unique DNA profiles. Microsatellites can consist of various types of repeats motifs, e.g. mononucleotide, dinucleotide, trinucleotide and tetra-nucleotide repeats. Since microsatellites have high mutation rates, these loci are mostly polymorphic which makes them ideal for determining gene flow between populations (Ellegren, 2004). Microsatellites can also be used to determine genetic distance between two populations (Ellegren, 2004; Luo *et al.*, 2007); as well as determining linkage mapping (including linkage-disequilibrium mapping) (Ohashi & Tokunaga, 2003), paternity testing, detection of genetic diseases and for forensic cases (Ellegren, 2004).

Cross-species markers are taken directly from closely related species and can assist in molecular analysis of species that is sparsely studied or where no molecular studies have been performed. These markers are also a cost-effective method since multiple species can be studied using the same marker. However, the success rate of these markers decreases with increased differentiation between species (Barbara *et al*., 2007). Therefore, it is preferred to use species-specific markers if possible. In pangolin, Luo *et al.* (2007) compared three pangolin species (*M. javanica*, *M. pentadactyla* and *P. tricuspis*) to determine the phylogeography, evolutionary- and population genetic status between these species. The authors designed 32 species-specific microsatellites markers for the Malayan pangolin (*M. javanica*, Asia). These 32 markers were then used as cross-species markers for the Chinese pangolin (*M. pentadactyla*, Asia) as well as for the white-bellied pangolin (*P. tricuspis*, Africa). The results indicated that 27 (84%) of the 32 markers amplified successfully for the Chinese pangolin and 18 (52%) for the white-bellied pangolin, which are more distantly related. The results provided new data on the current molecular status of these species by establishing how genetically different the three species were from each other. The results also indicated an average heterozygosity ranging from 0.321 to 0.708 for the three pangolin species.

The study performed by Luo *et al.* (2007) thus confirmed that cross-species markers are a valuable source where there is limited information available about a species, but that the quality and reliability of the results also decrease as the species become more genetically distant. Although the results attained from the cross-species markers in Luo *et al.* (2007) produced reliable results, the markers produced more consistent results when used as species-specific markers. Thus it could be advantageous in the future to develop species-specific markers for Temminck’s ground pangolin rather than using cross-species markers. To date, no studies using microsatellite markers on Temminck’s ground pangolin have been reported. However, an unpublished study by De Beer (2013) was performed, where 14 of the initial 32 cross-species markers from Luo *et al.* (2007) were tested on Temminck’s ground pangolin samples. The results produced were not robust and could thus not be used for reliable analysis.

1.4.2.2. SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) MARKERS

Single Nucleotide Polymorphisms are variations at individual nucleotide positions between species or paired chromosomes, which arise within a genome. The distribution of these nucleotide variations is not homogenous and they are more frequently found in non-coding regions compared to coding regions. Single Nucleotide Polymorphisms density can also be influenced by genetic recombination and mutation rate (Nachman, 2001). Currently, no published SNPs have been reported for any of the pangolin species in the family Manidae, both in Asia and Africa. Future research in the development of SNPs for pangolins could contribute to the identification of specific characteristics and measuring differentiation associated with gene regions, as well as identifying possible disease susceptibility (Nachman, 2001; Barreiro *et al.*, 2008).

1.4.2.3. Y-CHROMOSOME MARKERS

The sex-determining region Y (S*ry*) gene is a sex determining locus in mammals which occurs as a single copy and is located on the Y-chromosomes in males (Pamilo & O'Neill, 1997). This chromosome is paternally inherited and the Y-borne gene is responsible for determining maleness in Eutherian mammal species, as well as turning bipotential embryonic gonads into testis (Yu *et al.*, 2011). Using Y-chromosome markers in phylogenetic studies could assist in identifying and determining male-based migration patterns in mammals. The only Y-chromosome study performed on pangolins to date, was a study by Yu *et al.* (2011). The authors sequenced the S*ry* gene in the Formosan pangolin (*M. pentadactyla pentadactyla*) in order to determine the phylogenetic position of the order Pholidota in the Eutherian tree. The results indicated that the Formosan pangolin had a Laurasian origin and is closer related to the order Carnivora and Perrisodactyla compared to the order Xenarthra. The similarities between the order Pholidota and Xenarthra could be conscribed to convergent evolution, since both these orders are insectivores and they share similar morphological characteristics. Yet they are not closely related on a molecular basis (Yu *et al.*, 2011). No recorded Y-chromosome studies have been performed on any of the African pangolins' to date, including Temminck's ground pangolin.

1.4.2.4. MITOCHONDRIAL DNA MARKERS (MTDNA)

According to Castro *et al.* (1998), the mitochondrion is a free living organelle in the cell which is responsible for regulating metabolism and producing adenosine triphosphate (ATP) in organisms. These organelles can also replicate on their own and contain single-stranded DNA (ssDNA). Mitochondrial DNA can be used to describe the foundation for the usage of restriction analysis, evolutionary geneology and phylogeography of species, since it evolves rapidly and there is no recombination, thus yielding noticeable sequence heterogeneity within species (Avise *et al.*, 1979*a*; Avise *et al.*, 1979*b*; Allendorf *et al.* 2013). Mitochondrial DNA contains fewer repair enzymes to assist in the replication process; therefore it generally evolves at a faster rate compared to nuclear DNA. The average mutation rate in mtDNA is approximately five to ten times faster compared to nuclear DNA (Brown *et al.*, 1979).

Mitochondrial DNA is a circular molecule consisting of about 14.5–19.5 kilobases and it codes for 37 gene regions. These gene regions include between 20 and 30 transfer ribonucleic acid (tRNA) regions, two ribosomal ribonucleic acid (rRNA) regions, 13 protein coding regions (which includes cytochrome *c* oxidase, ATPase 6 and cytochrome *b*), as well as eight unidentified reading frames (Roe *et al.*, 1985; Castro *et al.*, 1998; Freeland, 2005; Ki *et al.*, 2010). Mitochondrial DNA is well suited for ancient or degraded samples since there is more mitochondria present in the cell, thus the increased chance of finding mtDNA compared to nuclear DNA. Furthermore, only a small amount of DNA is usually necessary for mtDNA testing, which widens the sampling prospects (Freeland, 2005).

Analysis of mtDNA is a very effective way to gain insights of a species’ evolutionary processes on a molecular level, as well as allowing insight into mitochondrial genome evolution. Mitochondrial DNA is frequently used in conservation genetics because it is an economically effective method to define the genetic structure of species not studied before (Galtier *et al.*, 2009). Several mtDNA genes can be studied using primer pairs that anneal to conserved areas. Mitochondrial DNA data can also be obtained through various more advanced techniques, such as the primer-walker method where a fragment cannot be sequenced in a single read and has to be divided into several smaller consecutive regions in order to obtain the whole fragment. This method is usually performed for whole genome sequencing as well as for large gene regions (Sanger & Coulson, 1975). Pyrosequencing is another method implemented to obtain partial sequence fragments, by sequencing ssDNA, using enzymatic properties, to synthesize a complimentary strand (Nyrén & Lundin, 1985). However, both these techniques are time-consuming, costly and labour-intensive. Next Generation Sequencing is a new cost-effective and less time-consuming method since a multitude of small fragments (of a genome) are simultaneously sequenced which permits assembling of the whole genome afterwards rather than undertaking region by region analysis as described above (Metzker, 2005; 2009).

Whole mtDNA sequencing is advantageous for use in genetic studies involving phylogenetics and haplotype distribution, as the whole genome contributes to more accurate results and conclusions from studies where different species are compared. However, individual gene regions are more advantageous when whole or partial gene regions are used for comparative studies between individuals or populations from the same species. Using multiple gene regions simultaneously is a faster method in obtaining accurate results compared to whole mtDNA genome sequencing which is more time consuming.

PARTIAL MITOCHONDRIAL DNA STUDIES: Zhang & Shi (1991) performed a study using 19 restriction enzymes targeting the mtDNA strand to determine whether the Chinese pangolin (*M. pentadactyla*) could be separated into three different sub-species. The authors concluded that there are three sub-species of *M. pentadactyla* and they were characterized as *M. p. pentadactyla, M. p. pusilla* and *M. p. aurita.* A study performed by Hsieh *et al.* (2011) observed 38 haplotypes in the Formosan pangolin (*M. p. pentadactyla*) using the D-loop gene region located in mtDNA to distinguish the level of differentiation between the Formosan pangolin and the black-bellied pangolin. The authors also detailed successful extraction of mtDNA from pangolin scale samples.

WHOLE MITOCHONDRIAL DNA STUDIES: The complete mtDNA genome of the black-bellied pangolin, *P. tetradactyla* was sequenced by Arnason *et al*. (2002). The authors determined the placement of various mammals in the Eutherian group by performing a phylogenetic study using only whole mtDNA genomes. Included among these mammals was *P. tetradactyla* (Pholidota) (Figure 1.5). The results indicated that the order Pholidota formed a sister grouping with the orders Carnivora and Perrisodactyla, rather than with the order Xenarthra. The similarities between the order Xenarthra and Pholidota was concluded to be a result from convergent evolution rather than a recent common ancestor. Sequencing of the whole mtDNA genome of the Chinese pangolin *M. pentadactyla* has also been completed by Qin *et al*. in 2012. The authors compared the whole mtDNA genome of the two pangolin species *M. pentadactyla* (Asia) and *P. tetradactyla* (Africa) in order to describe differences in gene regions, genome length and structure between these two species.

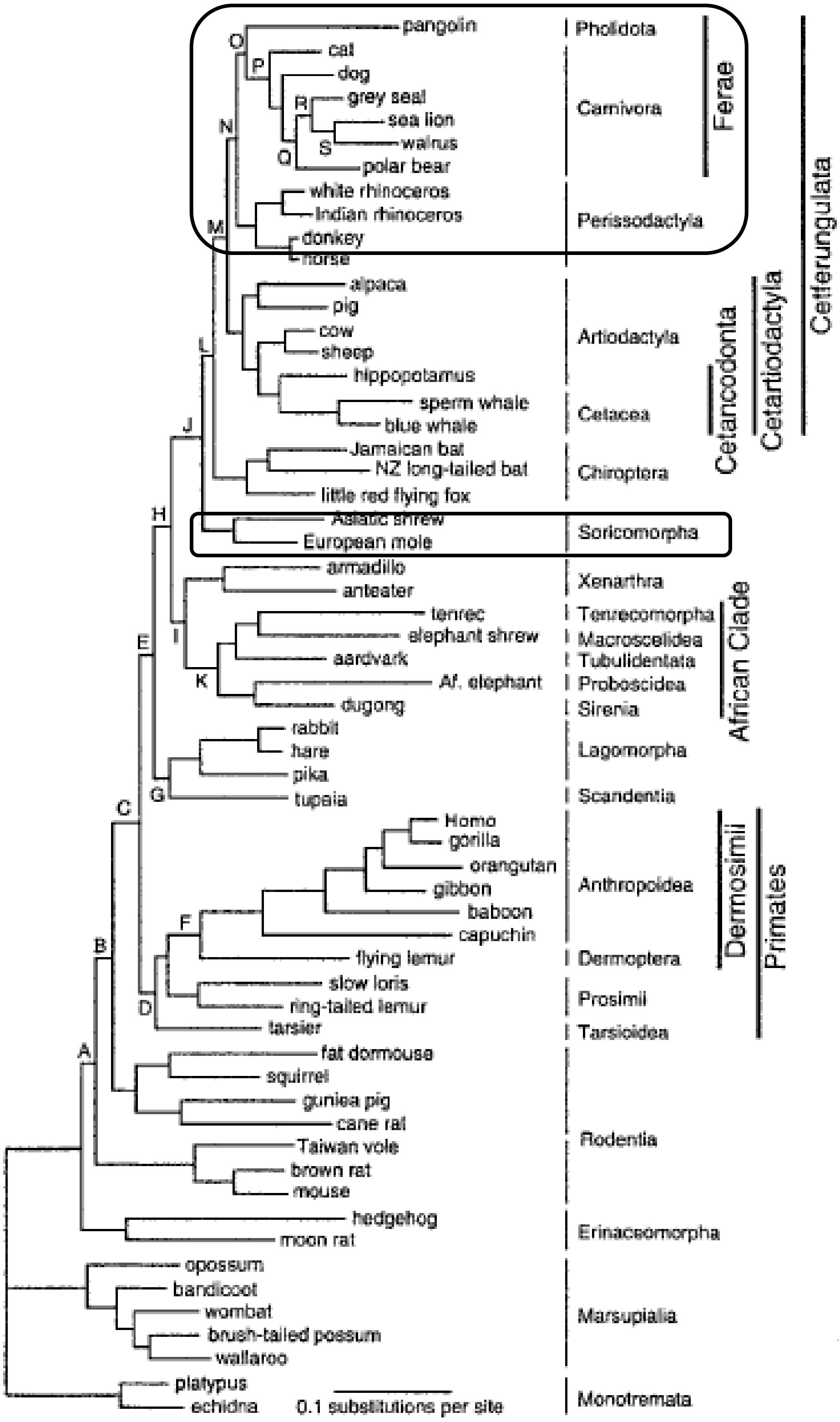

Figure 1.5: Phylogenetic tree illustrating the position of Pholidota in the Eutherian group, from the study by Arnason *et al*. (2002).

1.5. AIMS AND OBJECTIVES

1.5.1. AIMS OF THE STUDY

The overall aims of this study were to develop a molecular method to use whole mitochondrial DNA genome markers to gain insight into the population structure of Temminck's ground pangolins in southern Africa. The study presented here forms part of a larger project aimed at producing molecular data on all four of the African species, in order to contribute to the formulation of an effective conservation strategy for these animals.

1.5.2. OBJECTIVES OF THE STUDY

a) Optimize DNA extraction and Polymerase Chain Reaction (PCR) amplification of scale samples from Temminck's ground pangolin.

b) Sequence the whole mitochondrial DNA of Temminck's ground pangolin using cross-species and species-specific markers in order to determine the phylogenetic position of the order Pholidota in the super order Eutheria and determine the last common ancestor of the order Pholidota and the time of divergence from their closest relative.

c) Sequence three specific gene regions in the mitochondrial DNA of Temminck's ground pangolin, specifically cytochrome *c* oxidase I (COI), cytochrome *b* (Cyt*b*) and the D-loop. Determine haplotype distribution, population structure and genetic diversity for Temminck's ground pangolins using samples obtained from four countries in southern Africa, namely: Namibia, Zimbabwe, Mozambique and South Africa.

1.6. STRUCTURE OF THE DISSERTATION

This dissertation is presented in publication format, with papers (submitted for publication) covering various aspects of the study. Additionally, these papers are preceded by a general introduction (Chapter one) and the results discussed in a concluding chapter (Chapter six) to summarize and synthesize the findings of this research project. The papers submitted to international peer review journals are as follows:

1. Du Toit, Z., Grobler, J.P., Kotzé, A., Jansen, R. and Dalton, D.L. *Submitted: February 2014*. Scale samples from Temminck's Ground Pangolin (*Smutsia temminckii*); a non-invasive source of DNA. *Conservation Genetics Resources*.

2. Du Toit, Z., Grobler, J.P., Kotzé, A., Jansen, R., Brettschneider, H. and Dalton, D.L. 2014. The complete mitochondrial genome of Temminck's Ground Pangolin (*Smutsia temminckii*; Smuts, 1832) and phylogenetic position of Pholidota (Weber, 1904). *Gene* 551: 49-54.

3. Du Toit, Z., Grobler, J.P., Kotzé, A., Jansen, R., Pietersen, D.W. and Dalton, D.L. *Submitted: October 2014*. Molecular phylogeography of Temminck's Ground Pangolin (*Smutsia temminckii*) based on mitochondrial DNA variation. *African Zoology*.

CHAPTER TWO

ISOLATION AND CHARACTERIZATION OF MITOCHONDRIAL DNA MARKERS FOR THE TEMMINCK'S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*)

2.1. ABSTRACT

Poaching and habitat loss are severely depleting both African and Asian pangolin populations throughout their range and, consequently, pangolins are considered among the most threatened species of mammals in Asia (Newton *et al*., 2008) and Africa (Bräutigam *et al*., 1994). All eight pangolin species statuses have been uplisted on the IUCN Red Data List and range from *Critically Endangered* and *Endangered* to *Vulnerable* to extinction in some species. Temminck's ground pangolin (*Smutsia temminckii*; Pholidota) is no exception as they are harvested for bush meat and traditional medicine in Africa (Bräutigam *et al*., 1994) and are exported to Asia as a delicacy, as well as for medicinal uses (Newton *et al*., 2008; Challender & Hywood, 2012). Molecular data is very important to determine the genetic structure of a species. Mitochondrial DNA can be used to trace ancestral lineages of species and can further assist in determining the most effective conservation strategy for these endangered animals. During this study, 15 cross-species and 48 species-specific markers were designed to assist in the sequencing of the whole mtDNA of Temminck's ground pangolin. The use of cross-species markers designed from related species in combination with species-specific markers is an accurate and effective method to sequence whole mitochondrial DNA genomes. However, it is a time consuming process, thus other methods should be investigated for future research to contribute in this matter e.g. techniques such as next generation sequencing.

KEYWORDS: cross-species markers, species-specific markers, *Smutsia temminckii*, Temminck's ground pangolin, whole mitochondrial DNA

2.2. INTRODUCTION

The Temminck's ground pangolin (*S. temminckii*) is one of four extant species of pangolins found in Africa (Herklots, 1937; Gaudin & Wible, 1999; Hsieh *et al.*, 2011). Its distribution range extends from southern Africa through to northeast Africa (Chad) (Heath, 1992; Skinner & Chimimba, 2005; Soewu & Ayodele, 2009). Temminck's ground pangolin is currently listed as *Vulnerable* on the IUCN Red Data List for endangered species (Pietersen *et al*., 2014*b*). The biggest threat currently to the species is mortality from electric fences forming the perimeter of wildlife reserves, particularly in southern Africa (Pietersen *et al*., 2014*a*) and over-exploitation as a product in the bush-meat trade and *Muthi* markets (Sodeinde & Adedipe, 1994; Soewu, 2008; Soewu & Adekanola, 2011). Thus far, full mtDNA sequence data has been published for the black-bellied pangolin (Africa) *Phataginus tetradactyla* (AJ421454, Arnason *et al*., 2002) and the Chinese pangolin (Asia) *Manis pentadactyla* (NC016008, Qin *et al*., 2012) but no genetic studies have been reported for the full mtDNA sequence of the Temminck's ground pangolin to determine its genetic structure and phylogeny. The identification of primers for full mtDNA sequencing of this species is thus an important step in understanding the evolutionary relationship and phylogeny of this little known order. Furthermore, mtDNA analysis of this species can be used to identify population sub-structure and gain insights into evolutionary and phylogenetic relationships between the Temminck's ground pangolin and other pangolin genomes (Crozier, 1997) both in Africa and Asia. Analysis of mtDNA can be used to determine population structure within species (Moritz, 1994). This information in turn can be used to determine an individual's population of origin which can contribute to successful reintroductions (Freeland, 2005) as well as in forensic analysis as pangolins or pangolin body parts are often found in the illegal wildlife trade. Mitochondrial DNA sequencing can also be used to resolve phylogenetic relationships between species (Moritz, 1994). However some mtDNA studies are limited as they only focus on a few genes or regions of the genome, whereas whole genome sequencing can provide insights into genome organization (Boore *et al*., 2005). In addition, full genome analysis provides a higher resolution and power for more accurate phylogenetic analysis. Full mtDNA analysis is currently performed via designing primers from highly conserved regions of the genome using publically available complete sequences (Larkin *et al*., 2007) or with the use of primers designed from a closely related species or via cloning.

2.3. MATERIALS AND METHODS

Initially, 15 cross-species markers, of approximately 1000 bp each, were designed (Table 2.1) using the published whole mtDNA sequence of the black-bellied pangolin in order to sequence the complete genome of the Temminck's ground pangolin. Markers were optimized using a tissue sample and a scale sample obtained from a pangolin found by the APWG. The individual was found next to the road in the Northern Province of South Africa and suspected to be a road-killing accident as the cause of death was unknown. DNA extraction for both samples was performed by using the ZR Genomic DNA^{TM}–Tissue MiniPrep Kit (Zymo Research Corporation), following the manufacturer's protocol for solid tissue as well as the alternative protocol for hair, feathers or related samples for the scale sample. The quality of the samples was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Lithuania) and the DNA stored at -20˚C. Polymerase Chain Reaction amplification was performed using 2x Green DreamTaq^{TM} PCR Master Mix (Thermo Scientific, Lithuania) using the following reaction mix: 9.5 µl ddH$_2$O, 12.5 µl 2x DreamTaq^{TM} Mastermix, 1 µl of each 10 µM primer and 1 µl DNA to obtain a total reaction volume of 25 µl. Thermal cycling consisted of: initial denaturation at 95°C for 5 minutes, for 45 cycles, denaturation at 95°C for 30 seconds, annealing at 50–55°C for 30 sec, extension at 72°C for 45 sec, followed by final extension at 72°C for 7 min. Amplification was verified by running the PCR product on 2% agarose gels and the exosap (Thermo Scientific, Lithuania) protocol was used to purify the samples prior to cycle sequencing. Cycle sequencing was performed using the BigDye v3.1 Terminator Kit (Applied Biosystems, Foster City, CA) and the samples purified with a ZR DNA Sequencing Clean-up^{TM} Kit (Zymo Research Corporation) prior to sequencing on a genetic analyser. Purified PCR products were sequenced on a ABI 3130 genetic analyser (Applied Biosystems, Foster City, CA). The whole mtDNA of Temminck's ground pangolin was screened, aligned and analysed via CLC Bio Genomics work bench v5.0 software (CLC Bio, Aarhus, Denmark).

Table 2.1: List of 15 two directional cross-species markers used to sequence the whole mitochondrial DNA genome of the Temminck's ground pangolin (*S. temminckii*). The five markers indicated in bold were the only ones which did not yield any amplification for this specie.

Primer name	Primer Sequence	Fragment size Obtained (bp)	Annealing Temperature (˚C)
PNG_1	F: 5'- AGC TGG TAT CAA GCA CGC -3'	400-450	50
	R: 5'- GAA GGT AGC TCG TCT AGT TTC GGG -3'		
PNG_2	F : 5'- AAA GCA CCT GGC CTA CAC C -3'	200-280	50
	R: 5'- GCC CTC GTT TAG CCA TTC ATA C -3'		
PNG_3	F: 5'- ACC GTG CAA AGG TAG CAT AAT CA -3'	300-480	50
	R: 5'- ACG AGT TCT GAT TCT CCT TCT G -3'		
PNG_4	F : 5'- CCC CTA CCC ATA CCA TAC CC -3'	300-320	50
	R: 5'- GGG TTT ATT AGT ACG GGA AGG -3'		
PNG_5	F: 5'- GTA AGG TCA GCT AAA TAA GCT ATC GGG C -3'	280-300	50
	R: 5'- TAC TTG CTT AGG GCT TTG AAG GC -3'		
PNG_6	F: 5'- TAC GCC TAA CCT ACT CAA C -3'	-	-
	R: 5'- ACT TTT ACG CCT GTT GGG -3'		
PNG_7	F: 5'- CGA GCT TAT TTT ACA TCC GC -3'	550-600	50
	R: 5'- CTG TGT ATT CGT AGC TTC AG -3'		
PNG_8	F: 5'- ACG CCC AAG AAG TAG AGA C -3'	-	-
	R: 5'- GTG AGT TTG TTG GTT CAT TAG G -3'		
PNG_9	F: 5'- ATC AGC CTA CTT ATC CAA CC -3'	350-400	45
	R: 5'- GAG CCT CAT CAA TAA ATG GAG AC -3'		
PNG_10	F: 5'- CAC TTC GTA GAT GTA GTC TGA -3'	100-140	50
	R: 5'- TTC TAC GTG AGC TTT GGG -3'		
PNG_11	F: 5'- CGA TGA GGC AAC CAA ACA GAA -3'	550-570	55
	R: 5'- GGT TCC TAA GAC CAA CGG A -3'		
PNG_12	F: 5'- GTA TGC AAG AAC TGC TAA TTC ATG C -3'	280-300	55
	R: 5'- CCC CCT ATT TTA CGG ATG TCT TGT TC -3'		
PNG_13	F: 5'- AGC CAA TTG GGC CTA ATA AT -3'	-	-
	R: 5'- CGG ATG TTT GTC ATT AGG TTC -3'		
PNG_14	F: 5'- GCC GCT GTA TAA CCA AAA AC -3'	-	-
	R: 5'- TTG TAT AGT ATG GGT GGA AGG G -3'		
PNG_15	F: 5'- CAT GAA ACA GGA TCC AAC AAT CC -3'	-	-
	R: 5'- ACT AAT CAG CCC ATG CCG -3'		

2.4. RESULTS AND DISCUSSION

As indicated in Table 2.1, only 10 of the 15 initial cross-species markers successfully amplified for the Temminck’s ground pangolin sample (Figure 2.1). The markers did not sequence 1000 bp fragments as expected but instead smaller fragments were obtained. The cross-species markers were initially designed to amplify the whole mtDNA of the pangolin since no reference sequences were available for the species. One of the possibilities for the failure of some of the cross-species markers could be that the targeted fragment was too large for amplification to occur; or due to significant nucleotide differences between the two species, resulting in lack of binding of the primers. Species-specific markers were subsequently designed from the sequences produced via the cross-species markers to fill the gaps.

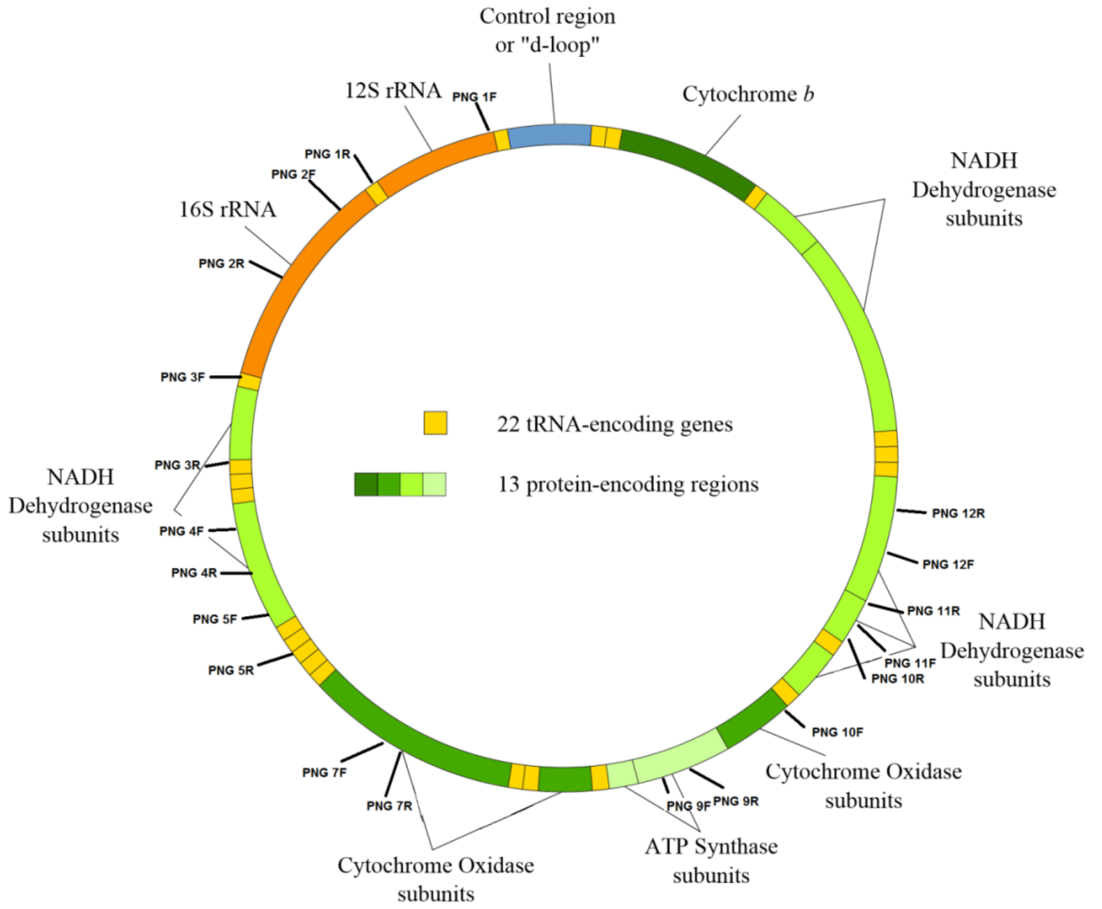

Figure 2.1: Diagram illustrating the main gene regions found inside the mitochondrial DNA (Kalicharan, 2011). It also depicts the various amplification regions obtained from the 10 cross-species markers used.

As illustrated in Table 2.2, forward species-specific markers in combination with reverse cross-species markers were used to amplify the larger regions that were still missing in the whole mtDNA genome of the Temminck’s ground pangolin.

Table 2.2: List of one-directional species-specific markers used to sequence the whole mitochondrial DNA genome of the Temminck’s ground pangolin (*S. temminckii*) to amplify longer regions.

Primer Name	**Primer Sequence**	**Fragment Size Obtained (bp)**	**Annealing Temperature (˚C)**	**Initial Amplification Section (bp)**
Pan1_intA	F: 5'- GCG GTC ATA CGA TTA ACC -3'	450-490	50	395-877
Pan1_intB	F: 5'- TGC TTC ATA TCC CTC TAG AGG AG -3'	450-490	50	675-1157
Pan1_intC	F: 5'- CTG AAT TAG GCC CTG AAG CAC GC -3'	350-390	50	949-1336
Pan3_intA	F: 5'- GAG TAA TCC AGG TCG GT -3'	480-520	50	2570-3083
Pan5_intA	F: 5'- CTT CAA TCG CCC ATC TAG G-3'	450-470	50	4499-4964

Thereafter forward and reverse species-specific markers were designed to fill further gaps and to ensure an overlap of more than 200 bp in length (Table 2.3). These markers were developed using the Temminck’s ground pangolin sequences obtained, as mentioned above. Species-specific markers amplified with higher success than the cross-species markers that were designed from the black-bellied pangolin.

Table 2.3: List of two directional species-specific markers used to sequence the whole mitochondrial DNA genome of the Temminck’s ground pangolin (*S. temminckii*) to amplify smaller regions.

Primer Name	**Primer Sequence**	**Fragment Size Obtained (bp)**	**Annealing Temperature (˚C)**	**Final Amplification Section (bp)**
Pan Pre-1	F: 5'- CAT CTT GTC AAA CCC CAA AAG C -3'	500-550	53	16292-302
	R: 5'- GGC ACG AGA TTT ACC AAC CCA T -3'			16304-282
Pan_Gap_2	F: 5'- CTC AGC AAA CAC AAG TCC CGC CTG T -3'	160-200	50	1973-2140
	R: 5'- AAA GCT CCA TAG GGT CTT CTC GT -3'			1902-2070
Pan_Gap_3	F: 5'- CTA CGT GAT CTG ATG TCA GAC -3'	270-300	50	2552-2829
	R: 5'- CCT ACA ATG TTT GGK CC -3'			2477-2750
Pan_4A	F : 5'- GTTGCCCAGACAATCTCCT -3'	320-360	50	3199-3551
	R: 5'- GGA ATC GTG GAT AGG ATG CTC GG -3'			3140-3484

Primer Name	Primer Sequence	Fragment Size Obtained (bp)	Annealing Temperature (˚C)	Final Amplification Section (bp)
Pan_Gap_4	F: 5'- AAT AGG CAT TGA ACC CAT ACC -3'	150-170	50	3826-3987
	R: 5'- TCG AAT CCA ACT CAG ATT GTC A-3'			3764-3826
Pan4C	F: 5'- CTC TGT GCT ACC AAA TAC ACC -3'	450-500	50	3877-4376
	R: 5'- CCT CCT CAC CCT CCA AT -3'			3804-4303
Pan_Gap_5B	F : 5'- GGA ATC CCA TTA TCA TCA GG-3'	300-330	50	4321-4634
	R: 5'- GAG GCC TCC TAT TGA CAT TAG G -3'			4505-4570
Pan_Gap_5C	F: 5'- CCT GCG TCT AAC CTA TTC CA -3'	200-240	50	4836-5070
	R : 5'- ACC AAT CTA GTG GGG ATT TAG C -3'			4779-5013
Pan_6A	F : 5'- TTA GGT TAC AAC TAG ACC AAG AGC -3'	400-500	50	5007-4515
	R: 5'- AAG GTT CCG GGT TGG CCT AAT TCA -3'			4975-5368
Pan6_intA	F : 5'- TCA GCC ACC TTA CCT ATG TTC -3'	380-430	50	5324-5750
	R : 5'- GAT GAG ATA CCC GCT AAA TG -3'			5308-5732
Pan_6B	F: 5'- GCT GGA ACT GGC TGA ACT GTA -3'	390-440	53	5741-6156
	R: 5'- TTA CGC TGC CTC CAT GTA AGG -3'			5831-6225
Pan_6C	F : 5'- GCT GGA ACT GGC TGA ACT GT-3'	70-100	50	5741-5841
	R: 5'- TGA CTT ATA GCG GGA GGT -3'			5673-5747
Pan_Gap_6D	F: 5'- GGC TTT ATC GTT TGA GCA CAT C -3'	90-120	50	6227-6326
	R: 5'- AAG CCC TAA TGC TCA TAG TAG AG -3'			6171-6284
Pan7_IntA	F: 5'- CAC ACG AGC CTA CTT TAC ATC AGC -3'	300-350	50	6260-6605
	R: 5'- TGC CCG AAA GAC CAA GGA AGT GTT G -3'			6210-6560
Pan_Gap_7A	F: 5'- CAA CGA CAC ATG AGC AAA AG -3'	50-90	50	6820-6895
	R: 5'- CAT AGG TAT GAT ATT GGC TTG -3'			6540-6593
Pan_Gap_7B	F: 5'- CAA TCT GAA CAG TTC TGC CTG C -3'	90-110	50	7243-7352
	R : 5'- TCA AAT GTC AGG TCT TCA TAG TCT G -3'			7184-7274
Pan8_intB	F: 5'- ATT CCC ATC ACC AAA ACG AC -3'	300-320	50	8055-8373
	R: 5'- CTA GGG CTA TGG GTT GAA TAA GC -3'			8014-8325
Pan_8A	F : 5'- CAA TCT GAA CAG TTC TGC CTG -3	370-420	50	7261-7677
	R: 5'- GGG TTC AGG TTT GTC CTT TAT AGC -3'			7669-8040
Pan_8B	F: 5'- CCT AGC AGT ACG ACT AAC CGC -3'	180-200	50	7995-818
	R: 5'- AAG GTT GGA GTT GCT ACG GTG AGG C -3'			7940-8120
Pan_8C	F : 5'- CCG TTC CTT TAT GAG CAG CAA CCG T -3'	160-200	50	8316-8476
	R: 5'- GAT AAA GGT TGG AGT TGC TAC GGT G -3'			8245-8405
Pan9_intA	F: 5'- CCG TAG CAA CTC CAA CCT TT -3'	380-450	50	8543-8984
	R: 5'- CGG ATG TAT TGA GTA GAG GTA C -3'			8508-8925
Pan_Gap_9A	F: 5'- GCA TGT AAT TAT CGG GTC TTC C -3'	100-140	50	9296-9398
	R: 5'- TGG AAG TTA ACT GTA CTG TG-3'			9210-9344

Primer Name	Primer Sequence	Fragment Size Obtained (bp)	Annealing Temperature (˚C)	Final Amplification Section (bp)
Pan_Gap_9B	F: 5'- CCT TTC ACA ATC TCC GAT G -3'	250-280	50	9215-9485
	R: 5'- GCA GGG ATG CTA GTA GTA CAT T -3'			9157-9430
Pan_10A	F: 5'- CCC TGC TTA TAC TGA TCG CCT T -3'	550-580	53	9560-10010
	R: 5'- GGC TAT GGG TTT GGC TCA GTA TT -3'			10541-10631
Pan_10B	F: 5'- ATC GCA CCT CAT ATC CTC AC -3'	540-580	50	10002-10573
	R: 5'- GGC TAT GGG TTT GGC TCA GTA -3'			10067-10614
Pan_11A	F : 5'- TGT TAC GTA TTA CTC CCC TAC TAG-3'	480-500	50	10951-11431
	R: 5'- AGG GCT ATT AGA GCA TTT TCT CG-3'			10889-11383
Pan_Gap_11	F: 5'- TTA CTC CCC TAC TAG AAC CTA CAA C -3'	460-510	50	10955-11460
	R: 5'- GGC AGT AGA TGG AGG GCT ATT -3'			10923-11388
Pan_11B	F: 5'- CCT CCA TCT ACT GCC TAT TCT -3'	260-300	50	11576-11843
	R: 5'- CAG TGC TTT ACA TAG ACT GGG TAG C -3'			11466-11734
Pan_Gap_12A	F: 5'- CCC TAA TAT GAC TCT CAC TCA CAA T -3'	190-210	50	11793-12003
	R: 5'- ACA AGG AGG GCT ACG GAT ATA A -3'			11746-11952
Pan_12A	F: 5'- GCT ACC CAG TCT ATG TAA AGA A -3'	400-440	50	11877-12312
	R: 5'- GGG TTA AGC GCA GAT AGC TGT -3'			12123-12550
Pan_Gap_12B	F: 5'- CCC AGT ATC AGC ATT ACT TCA CTC C -3'	100-130	50	12526-12648
	R: 5'- ATC CTA GTT GGC TTG AGG TTG A -3'			12454-12584
Pan_Gap_12C	F: 5'- TCA ACC TCA AGC CAA CTA GGA T -3'	130-150	50	12700-12850
	R: 5'- CGA TGA TAA GGG CGG AGG TT -3'			12639-12787
Pan_13N	F: 5'- CAA CCT CAA GCC AAC TAG GAT -3'	300-340	50	12723-13043
	R: 5'- ATA GCA CTT GGG GCG GCA AAC-3'			12649-12791
Pan_13A	F: 5'- TTA TAC AAA CGC CTG AGC CCT CTC -3'	330-360	50	13004-13351
	R: 5'- CTG AGG CTT GGG TTT CTA ATG TAG -3'			13141-13209
Pan_13B	F: 5'- GGC TAC TTC CCA ACA ATC ATC CAT CG -3'	400-450	50	13366-13808
	R: 5'- CTG AGG CTT GGG TTT CTA ATG TAG -3'			13347-13754
Pan_13D	F: 5'- CCA ACA CTA ACT CTA TTG CCG ACC C -3'	350-400	50	13806-14201
	R: 5'- TTG GAG GGG GTT GGG AGG TCA ATA A -3'			13759-14145
Pan_14A	F: 5'- CCA TAA ATA GGA GAA GGC TTA GAA G -3'	230-250	50	14043-14297
	R: 5'- GTG TTC TGC TGT GTA GTG TAT TGC -3'			13987-14228
Pan_14B	F: 5'- CCC TCC AAC ATC TCA GCA T -3'	360-400	50	14316-14703
	R: 5'- AGG GGT CGG AAT ATC ATA GTG CGT T -3'			14612-14981
Pan_14C	F : 5'- CCC CTC CAA CAT CTC AGC AT -3'	150-190	50	14274-14444
	R: 5'- CCG TAA TAT AAG CCT CGT CC -3'			14211-14398
Pan_14D	F : 5'- AAC CCC CTA AGC ACA CCT CCC CAT A -3'	320-390	50	14956-15338
	R: 5'- GGG CTT TAG TCT CCT TCC TGA GTC -3'			14980-15297

Primer Name	**Primer Sequence**	**Fragment Size Obtained (bp)**	**Annealing Temperature (˚C)**	**Final Amplification Section (bp)**
Pan_15N	F: 5'- AAG GAG ATT CTA ACC TCC CC -3'	330-370	50	15385-15745
	R: 5'- CCT TCA GTG GAG GTG ATA CG -3'			15331-15694
Pan_15A	F: 5'- CCA ACG GGC AAA TAC GCT ATG -3'	400-430	50	15526-15955
	R: 5'- GTC CTG CGA CCA TTG ACT GAA-3'			15639-16051
Pan_15B	F: 5'- GAC TGT GGG GTA GTT ATA GGA GAA -3'	450-490	50	15870-16328
	R: 5'- TTA GAG CGG GCA GAA AAC TG -3'			15844-16327

Cross-species markers are very effective for implementation where no genetic data is available for a given species, in order to obtain a basic understanding of their molecular make-up. However, species-specific markers will be required for full information and can be developed via the primer-walking method. The combination of species-specific and cross-species markers proved to be highly effective in accurately sequencing the mtDNA of the Temminck’s ground pangolin. It is nevertheless a very labour-intensive and time-consuming method. Alternative methods for future sequencing studies in pangolins could contribute to faster, more accurate sequencing of whole mtDNA genomes, potential alternative methods include Next Generation Sequencing (Metzker, 2010) and the use of alternative *Taq* protocols such as Expand long template PCR system (Roche, Boehanger Mannhein). The availability of full mtDNA genomes for genetic analysis can assist to implement better management strategies and conservation programs for these animals and support forensic cases where samples are confiscated from the illegal wildlife trade.

CHAPTER THREE

SCALE SAMPLES FROM TEMMINCK'S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*); A NON-INVASIVE SOURCE OF DNA

3.1. ABSTRACT

Invasive sampling is often stressful due to the capture and handling of animals. However, non-invasive sampling aims to obtain suitable samples without causing harm to the study organism, thus making it a more preferred sampling technique. Molecular methods have advanced in such a way that quality DNA can be obtained from less invasive mammal samples. Various PCR enhancing techniques have also been developed to assist in amplification of low quality DNA samples, to produce reliable results that can be used to monitor populations and species or for forensic applications. Temminck's ground pangolin is one of the few mammals to be covered in scales. In this study, we aimed to identify the most appropriate DNA isolation and PCR methods by testing five different isolation techniques as well as three different PCR methods and two different PCR additives. These various techniques were used used to improve isolation of DNA and downstream amplification of mtDNA from scale samples collected from pangolins. The results indicated that a combination of various DNA isolation techniques along with various PCR additives, BSA and glycerol, contributed to obtaining successful sequencing data for around 89% of scale samples. Thus, we conclude that it is possible to obtaining good quality results from scale samples.

KEYWORDS: DNA isolation, non-invasive sampling, PCR optimization, scale samples, Temminck's ground pangolin.

3.2. INTRODUCTION

There are numerous ways to collect molecular samples from animals; some of these are invasive while others are non-invasive to varying degrees. Invasive sampling techniques make use of tranquilisers, traps, handling of animals or even euthanization to obtain samples (Taberlet & Luikart, 1999). These techniques are sometimes necessary but can cause stress, pain and harm to animals. However, non-invasive sampling has become more popular since it is less stressful for the animal and samples can be obtained without harming the animal (Taberlet & Luikart, 1999; Bubb *et al.*, 2011). Temminck's ground pangolins' whole body is covered in overlapping scales (Herklots, 1937) which protects the animal from predators like lions (Wilson & Reeder, 2005) and pythons (Soewu & Ayodele, 2009) amongst others. When pangolins are threatened, they roll into a ball with the overlapping scales protecting the head and other vulnerable parts of the body (Herklots, 1937). This makes it very difficult to obtain blood or hair samples since the body is rolled too tightly. Furthermore, the scales are sharp which could cause injury when handled. Unrolling the animals to get blood samples is not advisable since it would cause unnecessary stress and trauma for the animal which could leave them vulnerable and easy prey (Pietersen, 2013). Thus, an alternative, less invasive sources of DNA that can be obtained from pangolins is their scales. Pangolin scales consists of keratin (Matsuda *et al.*, 2005), like human nails, therefore taking scale clippings is not harmful to the animal and it is a less stressful sampling alternative. In addition, scales would most likely be obtained in forensic cases where animal parts are obtained from the illegal wildlife trade, as scales are used for various traditional medicines in both Africa (Bräutigam *et al.*, 1994) and Asia (Newton *et al.*, 2008; Challender & Hywood, 2012).

Nuclear- as well as mitochondrial DNA can be found in all cells, from tissue and blood to skin, faecal samples, feathers and even scales (Taberlet & Luikart, 1999). Conservation genetics have become more advanced in the use of non-invasive samples where populations can be monitored and species identified (Taberlet & Luikart, 1999). Various population studies have been performed where hair (Morin *et al.*, 1993) and faecal samples in primates (Bubb *et al.*, 2011) were used to determine genetic diversity and population structure within and between different sub-populations. Scale samples have previously been used for pangolins by Hsieh *et al.*

(2011). The authors used confiscated scale samples for species identification of one of the Asian pangolins using forensic techniques. Despite the ethical advantages of non-invasive sampling, the use of such techniques for genetic analysis also has several disadvantages. These include very low quality and quantity of DNA, and in scales it could cause keratin inhibition. This in turn can affect further downstream methods, such as PCR and sequencing, may be more difficult to carry out (Taberlet *et al.*, 1996). Numerous DNA isolation kits and techniques have been designed to assist in the optimization of low quality DNA samples, for example, faecal kits are designed specifically for the isolation of DNA from faecal samples (e.g. ZR Faecal DNA MiniPrep kit, Zymo Corporation). There are also various PCR enhancing techniques to support the amplification of fragments or DNA sequences. These PCR enhancing techniques may, for example, consist of adding Bovine Serum Albumin (BSA) or glycerol to the PCR protocol to assist in amplifying the fragmented regions of mtDNA or nuclear DNA (Nagai *et al.*, 1998). Bovine Serum Albumin (Pääbo *et al.*, 1988; Kreader, 1996) is an enzymatic stabilizing agent which binds non-specifically to most PCR inhibitors. It has also been found to improve the yield of PCR amplification in samples that are difficult to amplify, such as faecal samples (Woide *et al.*, 2010). One of the disadvantages of BSA is its sensitivity to high PCR temperatures, which destroys its enhancing abilities (Farell & Alexandre, 2012). Glycerol has been used as a stabilizing reagent in PCR amplifications and this has been shown to improve denaturation of GC-rich DNA (Smith *et al.*, 1990).

The objective of the current study was to find suitable DNA extraction and PCR methods for mtDNA sequencing from pangolin scale samples. Our specific aim was to test various isolation protocols, PCR methods and additives to determine which method can be used to obtain the highest quality of DNA from the scale samples for successful downstream applications.

3.3. MATERIALS AND METHODS

3.3.1. SAMPLE COLLECTION

In this study, four pangolin scale samples were received from Namibia, two from Zimbabwe, two from Mozambique and one from the National Museum in Bloemfontein (South Africa), as noted in Chapter five. It was then attempted to amplify three gene regions of the mitochondrial DNA genome in these samples, namely cytochrome *c* oxidase 1 (CO1), cytochrome *b* (Cyt*b*) and D-loop.

3.3.2. EXTRACTION AND OPTIMIZATION

The scale samples were all pulverised to powder using a dental electric micro-motor drill (Zhengzhou Xinghua Dental Equipment, Henan, China). The amount of powder used for further analyses varied according to the different DNA extraction protocols used. During this study a total of five different extraction methods were used to determine which one resulted in best downstream mtDNA amplification using scale samples. The first method was based on the ZR Genomic DNA^{TM}–Tissue MiniPrep Kit (Zymo Research Corporation), used for scales as described for scales samples in Chapter five. The second extraction kit was the QIAamp$^{®}$ DNA Investigator Kit (Qiagen, Novato, CA), following the manufacturer’s protocol for hair and nail clippings. The third kit was the RecoverAll^{TM} Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA), following the manufacturers protocol for DNA extraction for tissue samples. The fourth kit was the MasterPure^{TM} Complete DNA and RNA Purification Kit (Illumina Company, Madison, USA) and the general protocol as suggested by the manufacturers were used. The last method consisted of the AutoMate Express™ Forensic DNA Extraction System (Applied Biosystems, Foster City, CA) along with the PrepFiler Express BTA Forensic DNA Extraction Kit (Applied Biosystems, Foster City, CA), using the manufacturer’s protocols. Following extraction, the concentration and quality of the extracted DNA was analysed on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Lithuania), and extracted DNA was stored at -20˚C until further usage.

3.3.3. POLYMERASE CHAIN REACTION (PCR) OPTIMIZATION

Polymerase Chain Reaction was performed using various enhancers to obtain high quality sequences, with low background peaks, from the scale samples. The first method used 2x DreamTaq^{TM} Mastermix (Thermo Scientific, Lithuania) and the protocol described in Chapter four with a three temperature thermal cycling program. This program consisted of: initial denaturation at 95°C for 5 minutes, followed 5 cycles denaturation at 95°C for 30 seconds, annealing at 50°C for 50 sec, extension at 72°C for 1 min; followed by a second step, for 15 cycles, of denaturation at 95°C for 30 sec, annealing at 48°C for 50 sec, extension at 72°C for 1 min; and followed by a third step of 20 cycles, with denaturation at 95°C for 30 sec, annealing at 46°C for 50 sec, extension at 72°C for 1 min; finally followed by final extension at 72°C for 5 min. The second method was performed with the Q-solution PCR protocol using the *Taq* DNA Polymerase Kit (Qiagen, Novato, CA) along with the following reaction mixture: 1.25 µl 10x PCR Buffer, 2.5 µl 5x Q-Solution, 2 µl (25 mM) $MgCl_2$, 1.3 µl (2 mM) dNTP, 1.5 µl of each (10 µM) Primer, 0.125 µl *Taq*, 0.35 µl ddH_2O and 2 µl DNA to a final reaction volume of 12.5 µl. The thermal cycle program as described above was used. A third enhancing method was performed by using the REPLI-g Single Cell Kit (Qiagen, Novato, CA) on the DNA isolated using the RecoverAll kit. Both a Q-solution and DreamTaq PCR were run with the REPLI-g-based samples, using the methods described above for each. In addition to various *Taq* reagents used, different enhancers at various concentrations were implemented in an attempt to obtain high quality sequences. A total of 0.25 µl–0.5 µl BSA (Thermo Scientific, Lithuania) was added to the DreamTaq PCR protocol (Chapter four) followed by PCR amplification using the three temperature cycling method. In addition, another enhancer was tested by adding only half of the ddH_2O with the other half replaced by 5–10% glycerol, in the DreamTaq PCR protocol (Chapter four), along with the three temperature cycle program mentioned above. All PCR products were visualized on 2% agarose gels in order to determine whether amplification occurred. Further downstream amplification and sequencing were performed using the same protocols and steps as described in Chapter four.

3.4. RESULTS

The various DNA extraction methods and PCR protocols attempted in this optimization study are detailed in the optimization protocol presented in Figure 3.1. As indicated in Figure 3.1, DNA was initially isolated using the Zymo kit and various PCR protocols were subsequently followed. Once successful sequencing results were obtained, per sample, no further alternative protocols were followed. However, if all PCR protocols failed to amplify the various mtDNA regions, the alternative DNA extraction kits were applied. In addition, the extracted DNA were each run with the above mentioned PCR protocols, with and/or without enhancers, to determine which overall method provided the best results.

Even though various methods were performed both for DNA isolation and PCR, some of the scale samples still did not yield successful sequences. This could be a result of degraded or fragmented DNA. The concentration and purity of DNA obtained from the various extraction kits used for the isolation of DNA from scale samples are presented in Table 3.1. For illustration during this study, four samples were used as representatives to indicate the success rate of the various extraction kits and PCR enhancing techniques used. As indicated in Table 3.1, the Qiagen, MasterPure, REPLI-g and PrepFiler kits produced high quality extracted DNA from the scale samples. Although the Zymo kit yielded poor DNA quality, downstream applications such as PCR and sequencing was still possible. The only isolation protocol that was not successful for any of the samples was the RecoverAll kit. Among the PCR protocols, the Q-solution protocol was the only *Taq* protocol that didn’t produce any results for any of the samples, even with the samples obtained using the REPLI-g Kit.

Table 3.1: DNA quality and quantity obtained for the four scale samples, from the six different extraction methods mentioned in the materials and methods. A260/A280 values between 1.7 and 2.0 were considered indicative of good DNA quality.

	A260/280 Value / Amount of DNA (ng/µl)			
Extraction Method	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Zymo Kit	1.38/3.49	0.29/0.25	0.52/0.66	1.75/2.65
Qiagen Kit	1.84/21.56	1.85/10.36	1.34/8.88	1.61/18.95
RecoverAll Kit	1.86/6.35	0.13/2.09	-0.13/0.96	1.64/17.55
REPLI-g Kit	1.87/435.32	1.88/398.23	1.89/283.61	1.88/360.31
MasterPure Kit	1.63/394.56	1.11/43.65	1.56/65.36	1.60/70.48
PrepFiler Kit	1.39/14.16	1.19/29.41	1.19/24.61	1.40/55.47

The remaining PCR techniques all produced sequences that were of high quality. The average length of high quality sequences obtained for the three gene regions ranged between 100–500 bp, depending on the respected primer amplification length (Figure 3.2). Successful sequencing was possible in ±89% (100/112) of the samples. Sequences were of high quality with low background amplification as indicated in Figure 3.2.

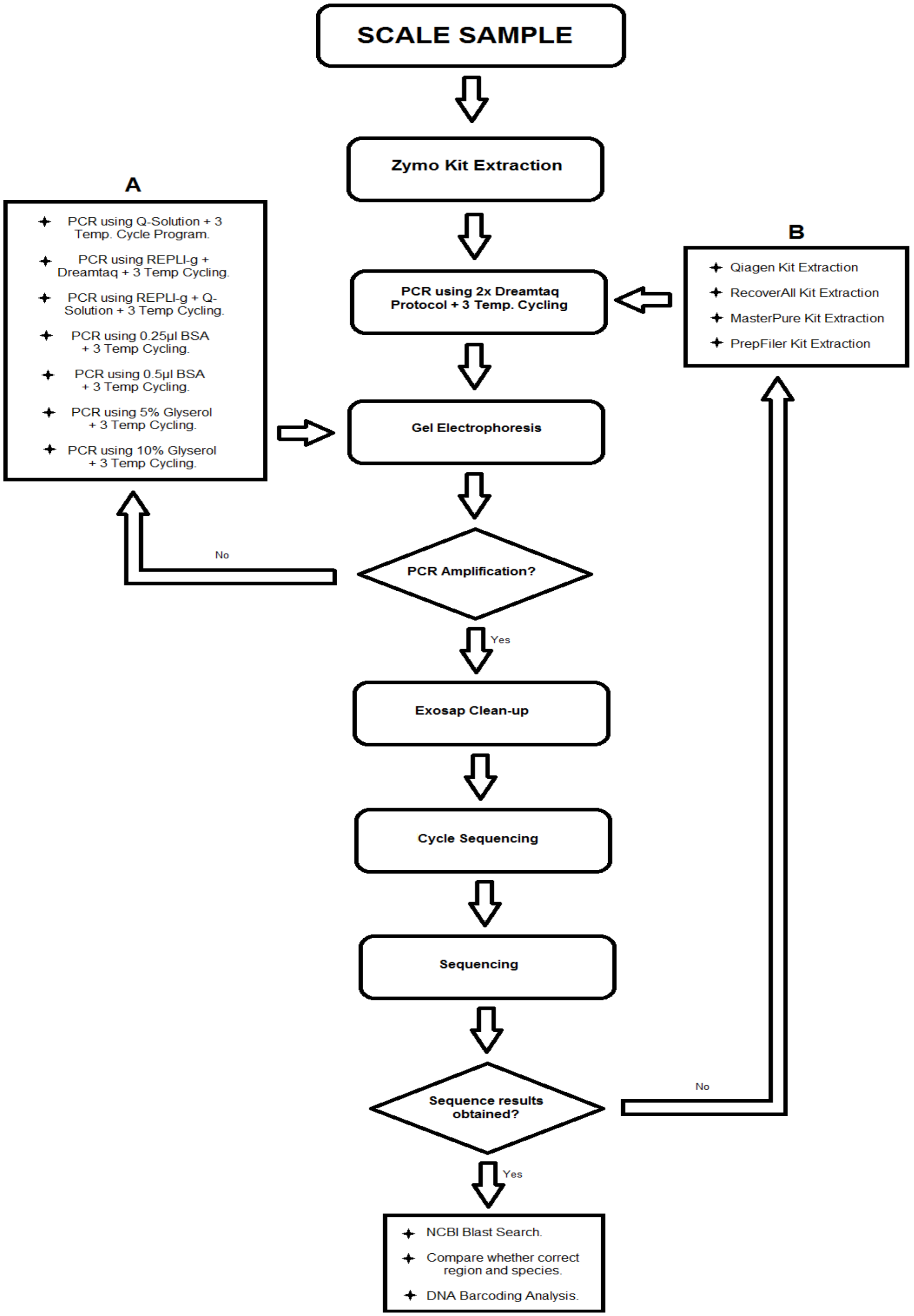

Figure 3.1: Optimization protocol indicating suggested steps in order to successfully obtain high quality sequencing results from mitochondrial DNA isolated from scale samples. (A) Details of *Taq* reagents and additives used during optimization. (B) Details of various DNA isolation kits used.

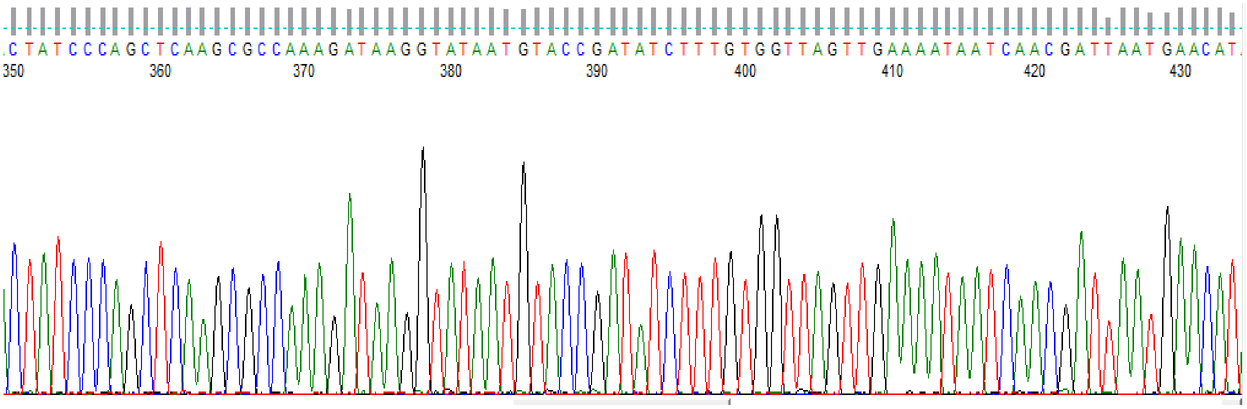

Figure 3.2: Example of high quality DNA sequence obtained from the COI gene region.

3.5. DISCUSSION

Various DNA isolation and PCR enhancer methods are available to enhance the quality of extracted DNA and sequencing results from degraded DNA samples. In this study, we obtained an overall success rate of about 89% with the amplification of scales samples after all five isolation techniques were performed with all eight PCR optimization techniques. From this, we conclude that it is challenging but nevertheless possible to isolate mtDNA from pangolin scale samples. Several different methods are required in order to successfully amplify DNA, and an optimisation protocol rather than a single method should thus be followed. The specific sample area of the scale however can also affect the success rate. Higher quality and quantity DNA is likely to be present at the base of the scale, where it is imbedded in the skin, than on the edges of the scales where it is easy to sample but had more time to degrade over time. Nevertheless, the optimization protocol presented here can be used to amplify gene regions from unconventional samples or low quality DNA from pangolins, for conservation research and for forensic applications. In addition, this technique may also be successful for other non-invasive samples such as faecal samples, shedded skin, hair or museum samples.

CHAPTER FOUR

THE COMPLETE MITOCHONDRIAL GENOME OF TEMMINCK’S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*; SMUTS, 1832) AND PHYLOGENETIC POSITION OF PHOLIDOTA (WEBER, 1904)

4.1. ABSTRACT

Temminck’s ground pangolin is primarily a nocturnal mammal belonging to the order Pholidota. The body is covered in hard overlapping scales and these animals find refuge in burrows, feeding only on termites and ants. In this study, the whole mitochondrial DNA of Temminck’s ground pangolin was sequenced and the phylogenetic position of Pholidota determined within Eutheria, using whole mitochondrial DNA sequences from various representative species. The results indicate that the whole mitochondrial DNA of Temminck’s ground pangolin is 16,559 bp long and shared some similarities with the whole mitochondrial DNA of the black-bellied pangolin and the Chinese pangolin. Phylogenetic analysis indicate that the order Pholidota is closely related and share a recent common ancestor with the order Carnivora rather than with the ant/insect eating order Xenarthra and the group Afrotheria. A time measured phylogeny of Pholidota estimated a split from Carnivora at around 87 million years ago (mya), followed by a split of the African pangolins from their Asian counterparts such as the Chinese pangolin at around 47 mya. This suggests a Laurasian origin and convergent evolution of the Pholidota with respect to Xenarthra and Afrotheria.

KEYWORDS: mitochondrial DNA, phylogeny, *Smutsia temminckii*, Temminck’s ground pangolin

4.2. INTRODUCTION

Mitochondrial DNA in mammals is a circular structure consisting of approximately 14.5 to 19.5 kilobases which codes for 37 gene regions. These gene regions include between 20 to 30 tRNA regions, two rRNA regions, 13 protein coding regions, various nucleotide gene regions which comprise of cytochrome *c* oxidase, ATPase 6 and Cyt*b* as well as eight unidentified reading frames (Roe *et al.*, 1985; Castro *et al.*, 1998, Freeland, 2005; Ki *et al*., 2010). Mitochondrial DNA is well suited for the analysis of ancient or degraded samples as it is preserved for longer compared to nuclear DNA and only a small amount of DNA is necessary for mtDNA sequencing (Freeland, 2005). Mitochondrial genome sequence and structure is widely used to provide information on phylogenetic relationships (Ermakov *et al*., 2006), to determine the genetic structure of populations and to assess patterns of gene flow (Avise, 1994).

Temminck’s ground pangolin (*Smutsia temminckii*; Smuts, 1832) is a mammal belonging to the family Pholidota (Weber, 1904). This is the only mammal species where the entire body is covered in hard overlapping scales (Herklots, 1937; Dollens, 2010) and they will roll into a ball when threatened to protect the inner softer parts of their bodies that are more vulnerable. These animals are predominantly nocturnal and find refuge in abandoned burrows made by other species, where they hide during the day (Heath, 1992). These animals only food source is ants and termites (Heath, 1992). They generally use their sharp claws to break open the termite nests (Herklots, 1937) and use their long proboscis tongue to remove termites from their tunnels (Heath, 1992). There are eight extant pangolin species, four of these occur in Asia and four in Africa (Herklots, 1937). All pangolin species are considered *Vulnerable*, *Threatened* or *Endangered* by the IUCN (IUCN, 2014). The four African species are further divided into two arboreal and two ground-dwelling species. Temminck’s ground pangolin has the largest distribution range of all four African species, ranging from southern Africa all the way to northeast Chad (Heath, 1992). To date, the only complete mitochondrial genome sequences available in the order Pholidota, is that of the Chinese pangolin (*Manis pentadactyla*) (Qin *et al*., 2012) and the black-bellied pangolin (*Phataginus tetradactyla*) (Arnason *et al*., 2002). Considering that full mtDNA sequencing is useful for obtaining more reliable phylogenetic data (Roe *et al.*, 1985; Freeland, 2005), the current study aimed to sequence the whole mtDNA genome of Temminck’s ground pangolin. We discuss results with respect to genome structure, gene arrangement, nucleotide composition, and codon usage.

Further, we report on phylogenetic analysis and molecular dating of Temminck's ground pangolin with the two pangolin species mentioned above in order to describe the phylogenetic relationships within the order Pholidota. We also describe the phylogenetic relationships between the order Pholidota with representatives from other ant-eating mammals from the order Eulipotyphla and Marsupialia as well as the closest related order Carnivora that has been identified previously in phylogenetic studies to be closely related to the order Pholidota (Arnason *et al*., 2002; Murphy *et al*., 2007). The out-group used during the phylogenetic analysis was representatives from the order Monotremata.

4.3. MATERIALS AND METHODS

4.3.1. SAMPLE COLLECTION AND DNA EXTRACTION

The specimen used to sequence the whole mtDNA of Temminck's ground pangolin originated from a deceased individual, collected by the African Pangolin Working Group (APWG) in the Kalahari region of South Africa. The cause of death was unknown, but suspected to be a road killing accident. A total of three muscle tissue samples were collected from various parts of the pangolin's body. Collected samples were placed in absolute ethanol for preservation and were stored at -20˚C until further usage. DNA extraction was performed by using the ZR Genomic DNA^{TM}–Tissue MiniPrep Kit (Zymo Research Corporation) using the protocol for solid tissue and following the manufacturers protocol. The quantity of the extracted DNA was determined by using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Lithuania) and the extracted DNA was stored at -20˚C.

4.3.2. PCR AMPLIFICATION AND SEQUENCING OF FULL MTDNA SEQUENCE

A total of fifteen primer pairs were designed based on the 16,571 bp reads identified in a previous study on the black-bellied pangolin (Arnason *et al*., 2002), to amplify fragments of approximately 1000 bp in length. Sequences obtained in this study were aligned to the full mtDNA sequence of *P. tetradactyla* in order to identify missing gaps. The missing regions were then filled using the primer walking method with species-specific markers, designed from the sequences obtained from Temminck's ground pangolin. Polymerase Chain Reaction (PCR) was performed using 9.5 µl ddH_2O, 12.5 µl 2x DreamTaq^{TM} Mastermix, 1 µl of each primer pair and 1 µl DNA to obtain a PCR reaction of 25 µl. The thermal cycling was conducted as follows: initial denaturation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 30 sec, annealing at 50–55°C for 30 sec, extension at 72°C for 45 sec, followed by final extension at 72°C for 7 min. Resulting amplicons were inspected on 2% agarose gels followed by purification using Exosap (Thermo Scientific, Lithuania). Purified templates were sequenced using the BigDye v3.1 Terminator Kit (Applied Biosystems, Foster City, CA), used according to the manufacturer's instructions and run with the ABI 3130 genetic analyser (Applied Biosystems, Foster City, CA). The ZR DNA Sequencing Clean-up^{TM} Kit (Zymo Research Corporation) was used to purify the sequences and remove excess products (BigDye and buffer) prior to genetic analysis.

4.3.3. SEQUENCE ASSEMBLY AND PHYLOGENETIC ANALYSIS

Sequences were viewed and edited using the Chromas programme embedded in MEGA v5.2 (Tamura *et al*., 2011). A sequence blast was done on the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nm.nih.gov/blast) to verify sequence identity. Assemblies, mapping and primer design were performed in CLC Bio Genomics Work Bench v5.0 (CLC Bio, Aarhus, Denmark) to obtain an overlapping view of the full mtDNA sequence from Temminck's ground pangolin in relation to the full mtDNA of *P. tetradactyla* (AJ421454) and *M. pentadactyla* (NC016008) for comparison and verification(Table 4.1). Final alignment of the whole mtDNA of Temminck's ground pangolin with that of the other species mentioned in Table 4.2 was performed using MAFFT v7 (Standley, 2013).

Phylogenetic analysis was performed using MrBayes v3.1 (Ronquist *et al.*, 2011) to establish relationships based on Bayesian Inferences (BI), while a Maximum Likelihood (ML) approach was implemented in PhyML v3.1 (Guindon *et al.*, 2010). MEGA v5.2 software was used to infer a Neighbor-Joining (NJ) tree while BEAST v1.7 (Drummond & Rambaut, 2007) software was used to infer a time-measured phylogeny and obtain estimates of divergence times for this dataset. The Akaike Information Criterion (AIC) in jModeltest v2.1.3 (Darriba *et al.*, 2012) was employed to determine the best fit model of sequence evolution. The same model was selected for BI, ML, NJ and BEAST analysis as it proved to be the best fit model calculated for each analyses. Nodal support for the NJ tree was evaluated through 10,000 non-parametric bootstrap replications, while ML analyses were carried out with 1000 bootstrap replications. The BI and BEAST calculations were run over two million generations, after which 25% of the trees were discarded as burn-in. The divergence time used to calibrate the BEAST tree was estimated by BEAST during the final run and the out-groups were both the orders Monotremata and Marsupialia. Tracer v1.5 (Drummond & Rambaut, 2007) software was used to assess trace files generated by MrBayes v3.1 and BEAST v1.7, in order to assess whether mixing was achieved and to choose a suitable percentage burn-in.

4.4. RESULTS

4.4.1. WHOLE MITOCHONDRIAL DNA (MTDNA) COMPARISON

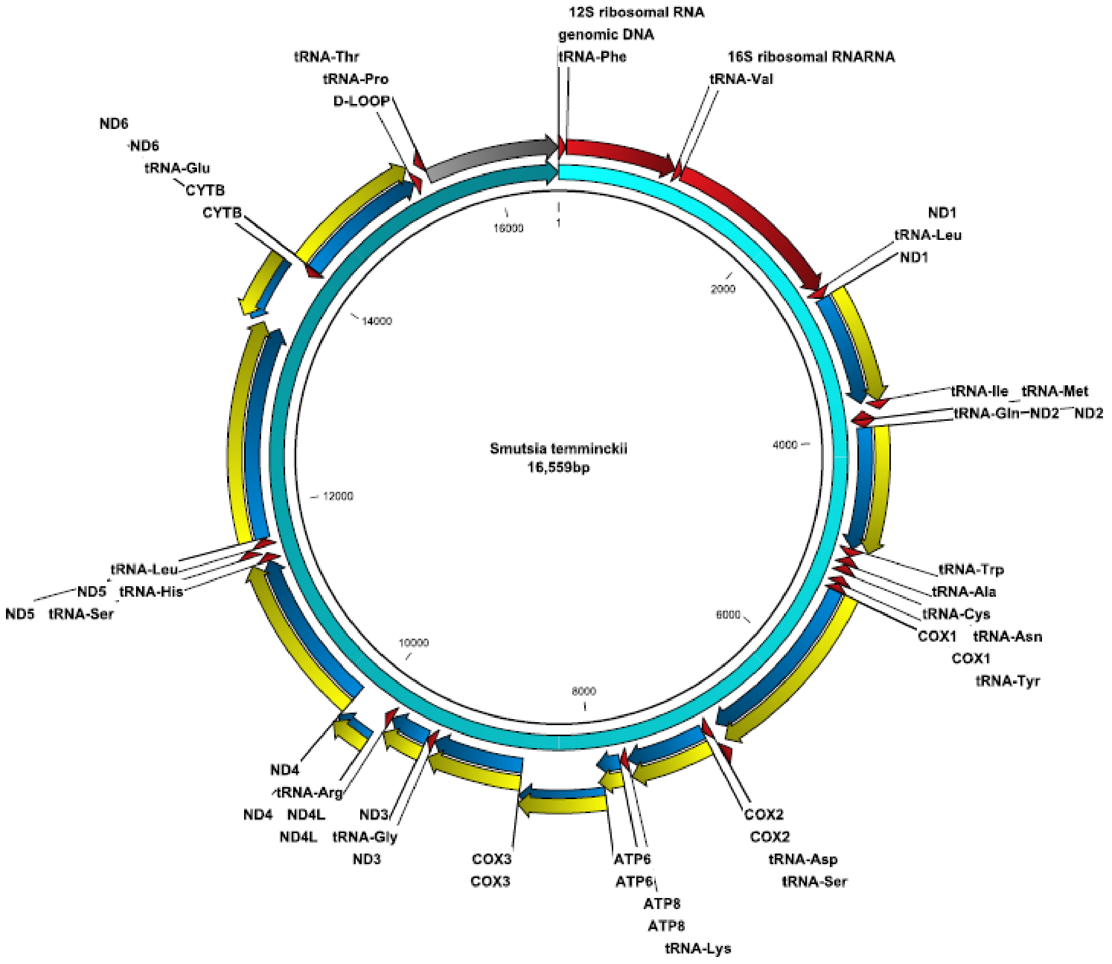

Figure 4.1: Circular representation of the whole mitochondrial DNA of Temminck's ground pangolin (*S. temminckii*) illustrating all the gene regions and various proteins associated with the mitochondrial DNA genome. Arrows indicate direction of transcription, where colours indicate the following: red = ribosomal DNA, blue = coding regions, yellow = genes and grey = non-coding region (D-loop).

The mtDNA genome of *S. temminckii* consists of 16,559 bp (Figure 4.1) compared to *P. tetradactyla* and *M. pentadactyla* which are 16,571 bp and 16,578 bp in length, respectively. As illustrated in Table 4.1, the mtDNA genome of *S. temminckii* varied from *P. tetradactyla* and *M. pentadactyla* in terms of gene region size at several genes. Two gene regions (16S and ND6) were found to be the same length in both *S. temminckii* and *P. tetradactyla* (Table 4.1) but with a different length in *M. pentadactyla.* Three gene regions (ND1, ATP8 and ND5) share the same length in both *S. temminckii* and *M. pentadactyla* and not in *P. tetradactyla*. In contrast, six gene regions (COX2, ATP6, COX3, ND3, ND4I and ND4) were found to be the same length in all three pangolin species and three gene regions (12S, ND2 and COX1) each have different lengths in all three pangolin species (Table 4.1). Only two gene regions (Cyt*b* and D-loop) were found to be of the same length in both *P. tetradactyla* and *M. pentadactyla* but different in *S. temminckii.* The AT content was determined for all three pangolin species and amount to 62.7% for *S. temminckii*, 63.1% for *P. tetradactyla* and 56.2% for *M. pentadactyla*. The nucleotide sequence similarity for the three species showed a 70.5% sequence similarity between *S. temminckii* and *P. tetradactyla.* Whereas the nucleotide sequence similarity between *S. temminckii* and *M. pentadactyla* amount to 69.9% and 70.8% between *P. tetradactyla* and *M. pentadactyla*. A previous study by Qin *et al*. (2012) found that there was a 79.3% similarity between *P. tetradactyla* and *M. pentadactyla* which separates them into different species levels; however the current study found that there was between 69.9% and 70.8% sequence similarity between the three pangolin species. Due to recent taxonomic changes in the order Pholidota, it would be more correct to indicate that the three pangolin species should be separated on a generic level rather than specific level. The difference in nucleotide similarity between the current study and the study performed by Qin *et al*. (2012) could be a result of different evolutionary models used or a different method of distance calculation between the two studies.

Table 4.1: List of mitochondrial DNA gene region sizes located in *S. temminckii*, *P. tetradactyla* and *M. pentadactyla.* The bold values indicate the gene region length shared between two pangolin species.

Gene Regions (bp)	***Smutsia temminckii***	***Phataginus tetradactyla* (Arnason *et al*., 2002)**	***Manis pentadactyla* (Qin *et al*., 2012)**
Entire Genome	16,559	16,571	16,578
12S rRNA	957	956	960
16S rRNA	**1553**	**1553**	1570
ND1	**955**	956	**955**
ND2	1041	1043	1038
COX1	1549	1553	1550
COX2	683	683	683
ATP8	**200**	206	**200**
ATP6	680	680	680
COX3	783	783	783
ND3	345	345	345
ND4I	296	296	296
ND4	1377	1377	1377
ND5	**1820**	1826	**1820**
ND6	**527**	**527**	524
Cyt*b*	1139	**1134**	**1134**
D-loop	1155	**1164**	**1164**

4.4.2. PHYLOGENETIC RELATIONS

The General Time Reversal model with invariant sites and gamma distribution (GTR+I+G model), with gamma parameter (G) = 0.74, was the best-fit model of sequence evolution for the dataset (Table 4.2) chosen under the AIC. Conserved sites for the nucleotide dataset amounted to 6221/16255, 9751/16255 which were variable and 8264/16255 parsimony informative. The transition:transversion ratio (R) was 0.96, while the proportions of nucleotides were T = 28.3, C = 25.1, A = 33.1 and G = 13.5.

Table 4.2: List of 22 species used for the phylogenetic analysis. The order, species and common name is indicated as well as the Genbank accession number.

Order	Scientific Name	Common Name	Genbank accession number
Monotremata	*Ornithorhynchus anatinus*	Platypus	NC000891
	Tachyglossus aculeatus	Short-beaked Echidna	NC003321
Marsupialia	*Didelphis virginiana*	Virginia Opossum	Z29573
	Myrmecobius fasciatus	Numbat	NC011949
	Dactylopsila trivirgata	Ant-eating Striped Possum	AB241054
Erinaceomorpha	*Erinaceus europaeus*	European Hedgehog	X88898
	Echinosorex gymnura	Moonrat	AF348079
Pholidota	*Manis pentadactyla*	Chinese Pangolin	NC016008
	Phataginus tetradactyla	Black-bellied Pangolin	AJ421454
	Smutsia temminckii	Temminck’s Ground Pangolin	Appendix A
Carnivora	*Panthera pardus*	Leopard	EF551002
	Acinonyx jubatus	Cheetah	NC005212
	Hyaena hyaena	Striped Hyena	JF894376
	Canis lupus	Grey Wolf	DQ480507
	Ailuropoda melanoleuca	Giant Panda	FM177761
	Lutra lutra	European Otter	FJ236015
	Arctocephalus pusillus	Brown Fur Seal	AM181018
Xenarthra	*Tamandua tetradactyla*	Lesser Anteater	AJ421450
	Dasypus novemcinctus	Nine-banded Armadillo	NC001821
Macroscelidea	*Macroscelides proboscideus*	Elephant Shrew	NC004026
Tenrecomorpha	*Echinops telfairi*	Lesser Hedgehog Tenrec	AJ400734
Tubulidentata	*Orycteropus afer*	Aardvark	Y18475

Separate NJ, BI and ML trees were constructed and due to similar topologies between the three trees; they were combined into one tree depicting each tree’s individual support values (Figure 4.2). Bootstrap values below 70% support were not indicated in the trees. Phylogenetic analysis of the three pangolin species favoured a monophyletic Pholidota that places the Chinese pangolin basal to the two African pangolin species. Maximum Likelihood analysis grouped the orders Pholidota and Carnivora with high support values (100%). Furthermore, the Xenarthra and Afrotheria formed sister clades as expected.

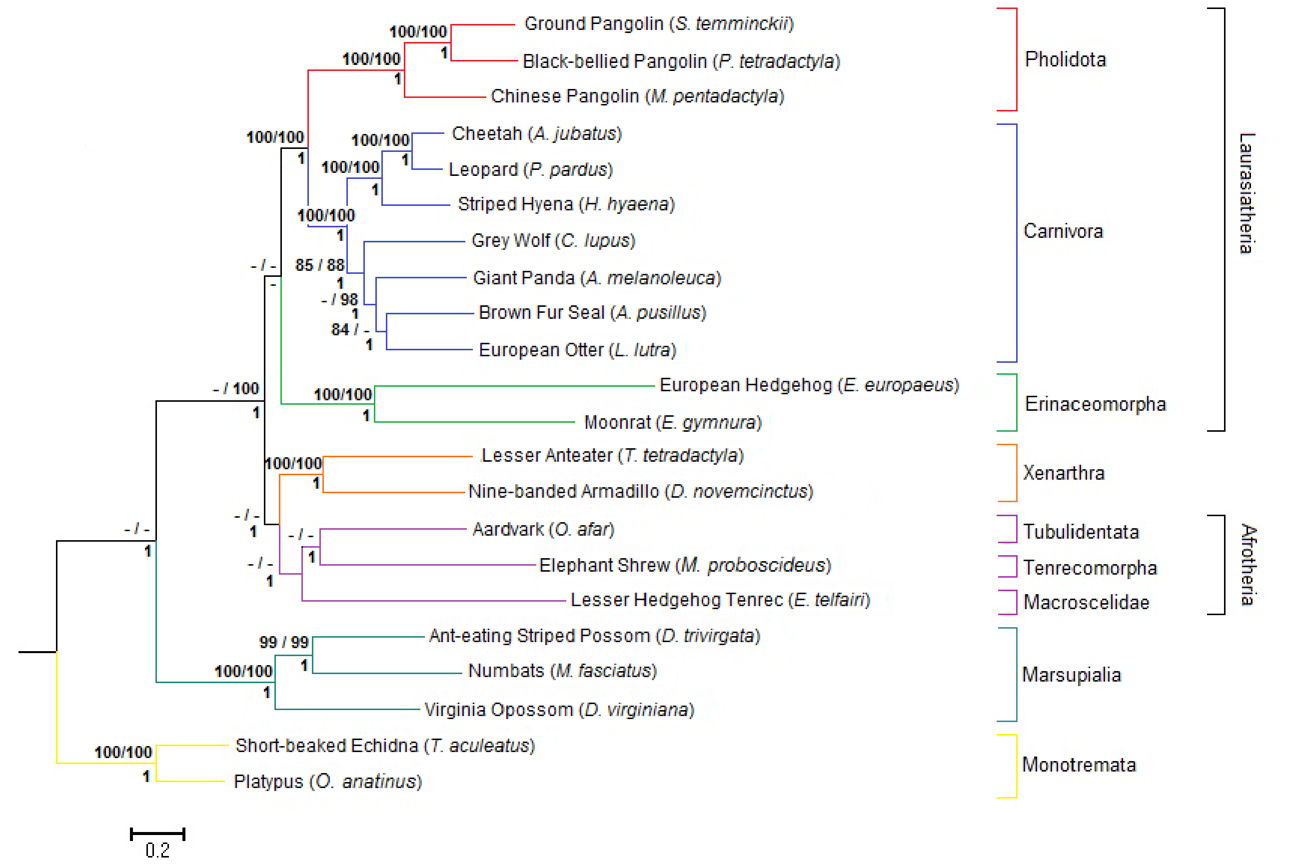

Figure 4.2: Phylogenetic tree comprising of combined Neighbor-Joining (NJ), Bayesian Inference (BI) and Maximum Likelihood (ML) using a GTR+I+G model for each tree. The NJ/ML bootstrap support is indicated above the tree branch and BI posterior probability support underneath the branch. Only values ≥70% was included.

Results from the BEAST analyses (Figure 4.3) supported the same relationships between taxa with node dates representing the time since last common ancestor or estimated divergence dates. Pholidota shared the last common ancestor with the order Carnivora about 87 mya and the two African pangolins split from the Chinese pangolin around 47 mya. In Figure 4.2, Erinaceomorpha clusters with Pholidota and Carnivora, forming part of the group Laurasiatheria whereas with the BEAST tree (Figure 4.3) they group separately.

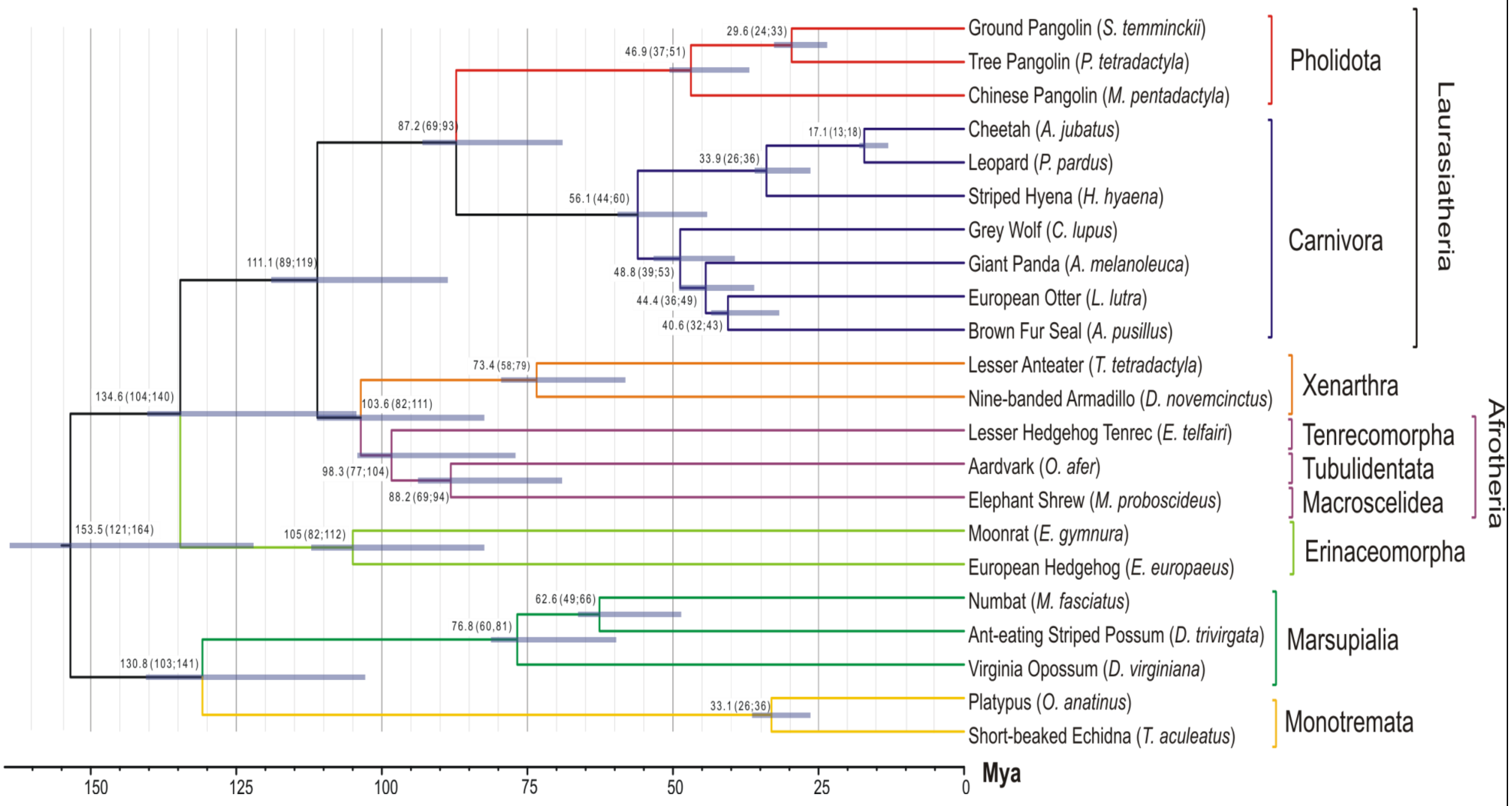

Figure 4.3: Phylogenetic tree illustrating divergence dates on the node of each branch. The dates on the nodes are indicated in million years ago (mya) since the last common ancestor was present or when the order split from the common ancestor. The node bars indicate 95% confidence intervals on the divergence time estimates expressed in mya before the present and the intervals for each date are presented in brackets.

4.5. DISCUSSION

4.5.1. PHYLOGENETIC RELATIONS AND ORIGIN OF PANGOLINS

Full mitochondrial genomes are available for two African pangolin species. In the wider dataset, the pangolins (Pholidota) form a monophyletic grouping with the order Carnivora. It would be expected that pangolins would group with the phenotypically similar order Xenarthra and the group Afrotheria. This Carnivora–Pholidota grouping is supported by an mtDNA study to determine the position of Eutheria (Arnason *et al*., 2002). In addition, molecular studies on partial regions of mtDNA and genomic DNA confirm the grouping observed during the current study (Springer *et al.,* 2004; Matthee *et al.,* 2007; Murphy *et al.,* 2007). The split among Xenarthra, Afrotheria and Pholidota is thus an example of convergent evolution (Reiss, 1997; Springer *et al.,* 2004; Matthee *et al.,* 2007 and Yu *et al.,* 2011) as Pholidota developed an ant-eating diet and have phenotypic characteristics similar to Xenarthra and Afrotheria, without these groups sharing the same direct common ancestor. As illustrated in Figure 4.2, Xenarthra and Afrotheria form a monophyletic clade, suggesting parallel evolution. In this study, Erinaceomorpha clusters with Pholidota and Carnivora, forming part of the group Laurasiatheria (Figure 4.2) whereas with the BEAST tree (Figure 4.3) they group separately. Previous studies however provide evidence that the grouping pattern illustrated in Figure 4.2 is the more supported pattern of differentiation (Arnason *et al*., 2002; Murphy *et al*., 2007).

According to the divergence dates illustrated in Figure 4.3, Laurasiatheria (Pholidota and Carnivora) shared a common ancestor about 87 mya and both orders originated in the super continent Laurasia when it separated from Pangaea (Figure 4.4). The group Afrotheria and order Xenarthra, on the other hand, cluster together even though Afrotheria originated in Africa and Xenarthra in South America. The shared common ancestor for both originated around 103 mya which correlates to the time when the supercontinent Pangaea splits into Gondwanaland (Figure 4.4) consisting of both South America and Africa. These observations were also observed in a study performed by Murphy *et al*. (2007) which confirm the dates found for Laurasiatheria, Xenarthra and Afrotheria. This provides further confirmation that Pholidota underwent convergent evolution as mentioned above.

Due to the basal clustering of the Chinese pangolin within Pholidota, it is deduced that Pholidota had their origins in Europe and Asia and later spread to Africa. Further evidence that favour this conclusion is that the oldest and most fossil pangolins were all found in Europe (Storch & Martin, 1994 and Gaudin *et al.*, 2009), Asia (Gaudin *et al.*, 2006) and North America (Emry, 2004), which all formed part of the super continent Laurasia between 200 and 120 mya. The results further indicate that the African and Asian pangolin species shared a last common ancestor about 47 mya indicating the effect of a natural occurrence, e.g. global cooling (Gaudin *et al.*, 2009) which may have interrupted gene flow between populations. This led to some members of the group ascending deeper into Europe and Asia, while others descended into Africa. Migration most likely led to specialised adaptations on each continent.

Temminck's ground pangolin and black-bellied pangolin both originate from Africa, and form a monophyletic group (Figure 4.2). The four African pangolin species are divided into two groups which consist of two arboreal and two ground-dwelling species. Since very little information is available on the other three African pangolin species, excluding Temminck's ground pangolin, it is difficult to determine which group evolved first. According to Gaudin *et al.* (2009), the oldest fossil of pangolins found in Africa was similar in appearance to the giant ground pangolin (*Smutsia gigantea*), a ground-dwelling pangolin species in Africa. Thus, it can be hypothesized that pangolins which descended into Africa were most likely ground-dwelling similar to the Asian species and migrated to the South–eastern parts of Africa to live in drier open habitats. Whereas, some animals adapted to arboreal lifestyles in forest habitats and most likely ascended into the trees due to better food availability, less competition as well as better protection against predators compared to burrows. However, additional research on phylogenetic analysis of African pangolin species should be conducted to support this hypothesis.

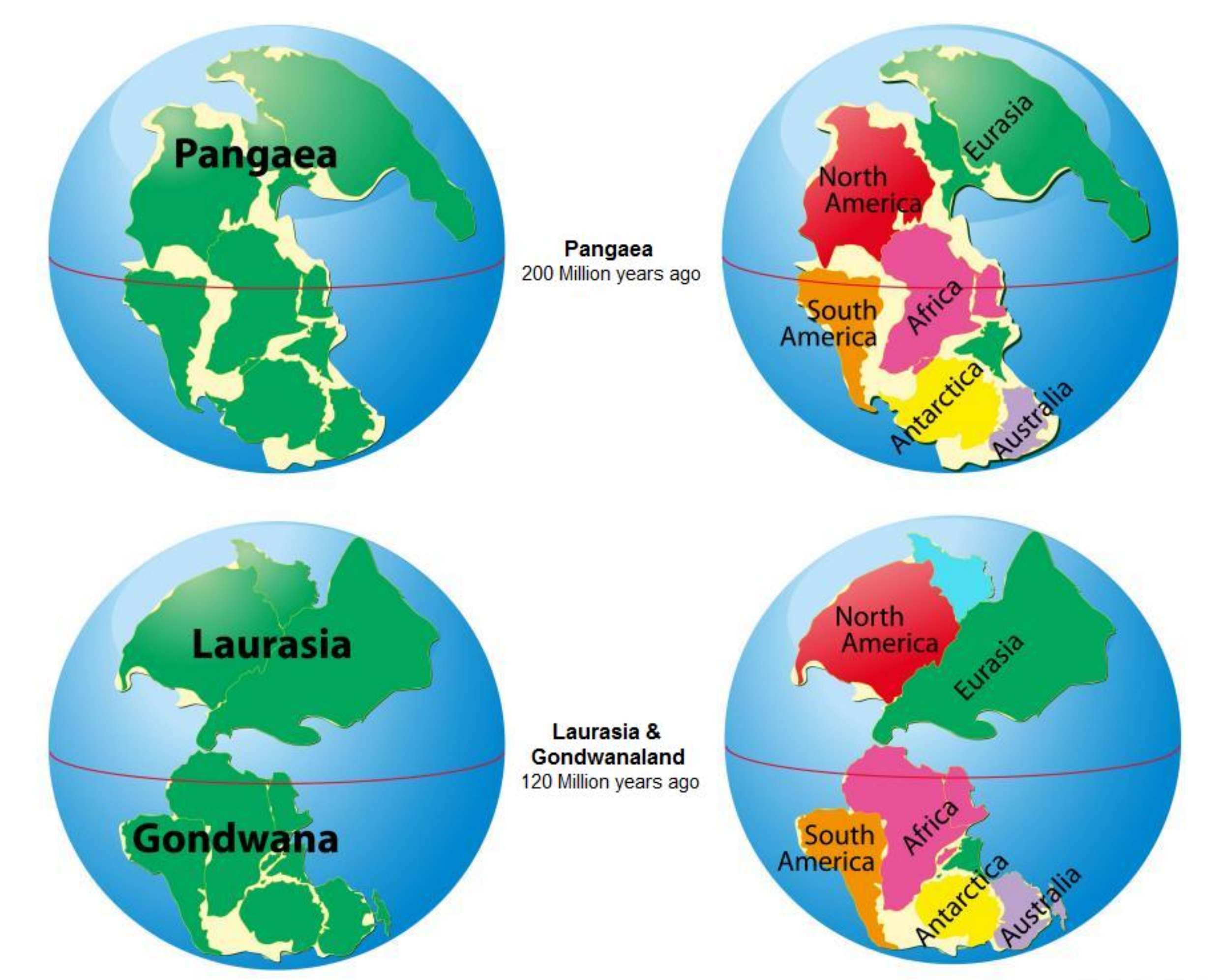

Figure 4.4: Supercontinent Pangaea illustrating the different positions of the present day continents. Pangaea split into two new super continents called Laurasia and Gondwanaland which moved to the respected poles and shows how the present day continents were affected with the break-up explaining continental drift and illustrates how speciation could occur in various species (Illustration: Anonymous, 2013).

CHAPTER FIVE

MOLECULAR PHYLOGEOGRAPHY OF TEMMINCK'S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*) BASED ON MITOCHONDRIAL DNA VARIATION

5.1. ABSTRACT

Temminck's ground pangolin faces numerous threats and is listed as *Vulnerable* by the IUCN. Some of the threats include over-harvesting for bush meat and it is used in traditional medicine. This study represents a first attempt to establish the molecular phylogeography of Temminck's ground pangolin across southern Africa. Unique haplotype distribution and phylogeography gave insights to how diverse the populations were. Furthermore, different groupings of Analysis of Molecular Variance (AMOVA) were performed to distinguish between and within different paired populations. The different pairings gave a better indication as to which, of these populations are connected or not. These analyses were performed using the three mtDNA gene regions CO1, Cyt*b* and D-loop. There was a high level of genetic variation within populations. The results indicated that samples from the Northern Cape and Mpumalanga Provinces of South Africa grouped with samples from Namibia, while samples from Zimbabwe and Mozambique grouped together. This separation could either be the signature of an ancestral or recent split. The BEAST analysis suggested that the two groups shared a recent common ancestor around 2.94–1.27 mya. A more recent split could be attributed to the Mega Kalahari Sand Sea forming a barrier between individuals and populations. The two splits identified in this study were estimated to have occurred between 920 and 710 kya for the Zimbabwe/Mozambique split and between 1.16 mya and 790 kya for the Kalahari/Namibia/Mpumalanga split.

KEYWORDS: cytochrome *c* oxidase I, cytochrome *b*, D-Loop, distribution, heredity, southern Africa

5.2. INTRODUCTION

Temminck's ground pangolin (*S. temminckii*) is one of eight extant pangolin species in the world and one of four extant species in Africa (Wilson & Reeder, 2005). This species has the largest distribution range of the four African species and is also the only pangolin species occurring in the southern African region. Their range extends from South Africa to north–eastern Chad (Heath, 1992; Skinner & Chimimba, 2005; Pietersen *et al.*, 2014*b*). They are found in arid and mesic savannah and dense woodland, but are absent from true desert and forest (Skinner & Chimimba, 2005). Temminck's ground pangolin is currently listed as *Vulnerable* on the IUCN Red List of Threatened Species (Pietersen *et al*., 2014*b*).

Local vulnerability is mainly due to over-harvesting of pangolin products such as meat and scales for the traditional medicine or *Muthi* trade in Africa (Chakravorty *et al.*, 2011; Soewu & Adekanola, 2011). According to Soewu & Ayodele (2009) the continued over-utilisation of Africa's pangolins for traditional medicine may contribute significantly to the extinction of these species in Africa. In more recent times, pangolins harvested in Africa are exported to Asia where they are highly sought after as a delicacy and where the scales and other body parts are used for various traditional medicinal purposes (Newton *et al*., 2008; Pantel & Chin, 2009; Soewu & Adekanola, 2011; Challender & Hywood, 2012). As this illegal trade increases, it is expected that larger amounts of pangolin body parts will be intercepted at customs points. Due to morphological similarities, body parts such as scales often cannot be linked to any one particular species and this creates a need for alternative testing methods to ascertain the species and its possible origin.

DNA analysis has been proven to be a useful tool in such cases and has played an important role in forensic cases where animal parts are confiscated and the origin of the animal traced to assist in identifying targeted areas for harvesting (Jun *et al*., 2011). In addition, some pangolins are confiscated alive and knowing their origin can contribute to their successful rehabilitation. The use of mtDNA markers has been reported in numerous genetic studies aimed at identifying species as well as determining population differentiation (Avise, 1994). Previous studies made use of CO1 to determine phylogeography in mice and other Eutherians (Luo *et al*., 2007; Violane *et al.*, 2012). The gene region Cyt*b* has also been used to investigate phylogeography in giraffes, *Giraffa camelopardalis* and African lions, *Panthera leo* (Burger *et al*., 2004; Dubach *et al*., 2005; Hassanin *et al.*, 2007) and the mtDNA control region (D-loop) used to determine phylogeography in warthogs, *Phacochoerus africanus,* African lions, various African bovids and red pandas, *Ailurus fulgens* (Arctander *et al*., 1999; Su *et al*., 2001; Muwanika *et al*., 2003; Barnett *et al*., 2006). This indicates that the use of these three mtDNA gene regions is a valid method for determining the phylogeography of species. Also the use of three independent gene regions serves to verify the results, by comparing the patterns observed across all three genes.

This study was the first attempt to determine the extent of genetic variation, haplotype diversity and phylogeography between different sub-populations of Temminck's ground pangolin across the southern African distribution range of the species. We also want to determine whether there was a genetic separation between the western and eastern parts of the distribution since morphological differences were observed in individuals from the eastern and western populations.

5.3. MATERIALS AND METHODS

5.3.1. SAMPLE COLLECTION, PREPARATION AND EXTRACTION

In this study, a total of 25 Temminck's ground pangolin samples collected from six regions in southern Africa were analysed (Figure 5.1). The type of samples analysed varied among sources and consisted of scales, tissue, skin or blood. The overall sample sizes consisted of four samples from Namibia (Nam), five samples from Zimbabwe (Zim), two samples from Mozambique (Moz), six samples from the Sabi Sands Private Game Reserve, Mpumalanga Province, South Africa (MP) and seven samples from Tswalu Kalahari Reserve, Northern Cape, South Africa (Kal). One museum sample was obtained from the National Museum in Bloemfontein (Free State Province, South Africa). This sample does not have any associated collecting details, but based on morphology and proximity of the specimen, it is believed to have originated from the Northern Cape Province where the population is most abundant. Sequence data from the black-bellied pangolin (*P. tetradactyla;* AJ421454, Arnason *et al*., 2002) was obtained from the NCBI nucleotide bank (Genbank) and used as an out-group in the analysis. Collected tissue and skin samples were placed in absolute ethanol, blood in Ethylene Diamine Tetra-acetic Acid (EDTA) tubes and scales in envelopes for preservation, and were stored at -20˚C until further usage.

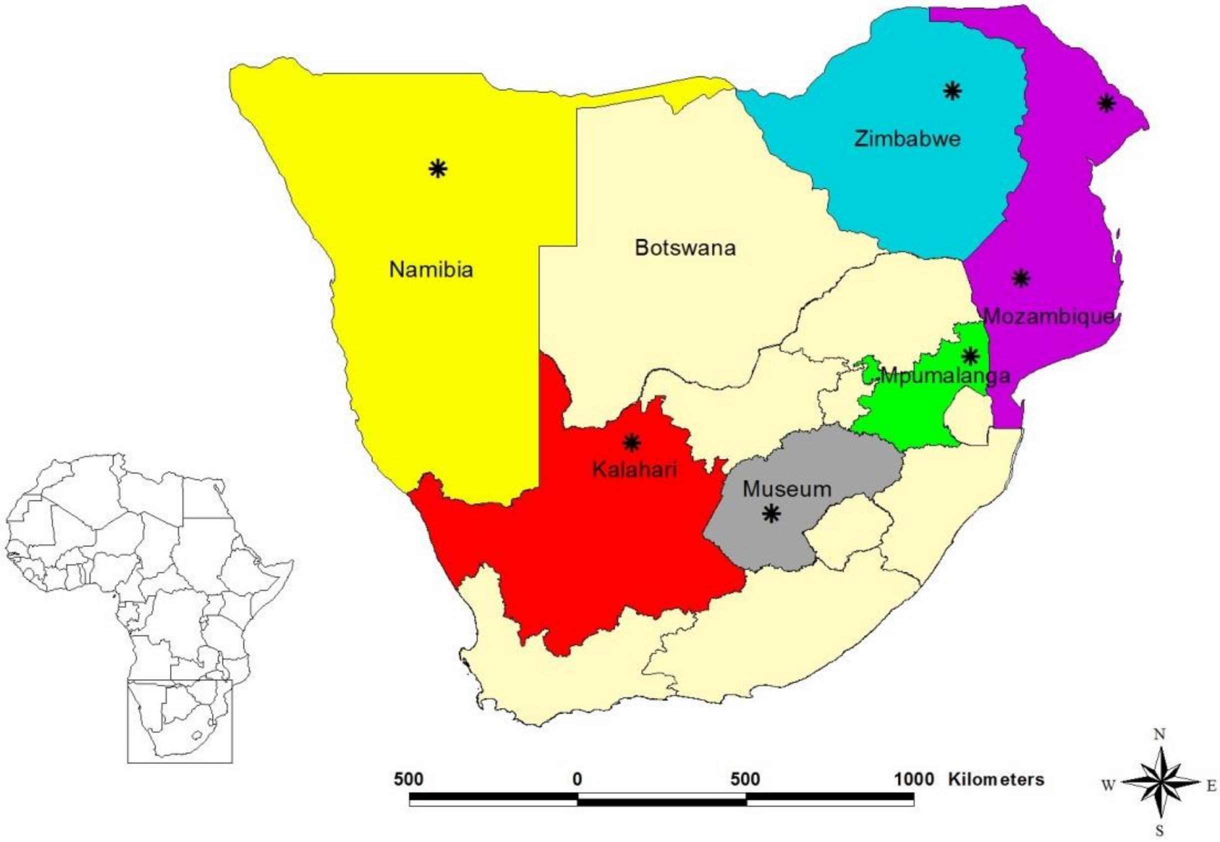

Figure 5.1: Sampling map illustrating the sampling areas used for the study: Tswalu Kalahari Reserve, Northern Cape, South Africa (Kal); Sabi Sands Private Game Reserve, Mpumalanga Province, South Africa (MP); Zimbabwe (Zim); Mozambique (Moz); Namibia (Nam) and the National Museum sample, Bloemfontein, Free State Province, South Africa. Map colours correspond to the colours used in the haplotype networks.

All samples were extracted using the ZR Genomic DNA^{TM}–Tissue MiniPrep Kit (Zymo Research Corporation), where the applicable protocols were used to extract DNA from tissue and blood samples. DNA from scale and skin samples was extracted by means of the alternative protocol for hair, feathers or related samples, with alterations. The digestion step included a mixture of 70 µl ddH_2O, 90 µl 2x Digestion Buffer, 20 µl DTT (1 M) and 20 µl Proteinase K. Samples were incubated for 48 hours in a heating block or until suspended properly. A NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Lithuania) was used to determine the quality and quantity of the extracted DNA and samples were stored at -20˚C until further usage.

5.3.2. POLYMERASE CHAIN REACTION (PCR) AND CAPILLARY ELECTROPHORESIS

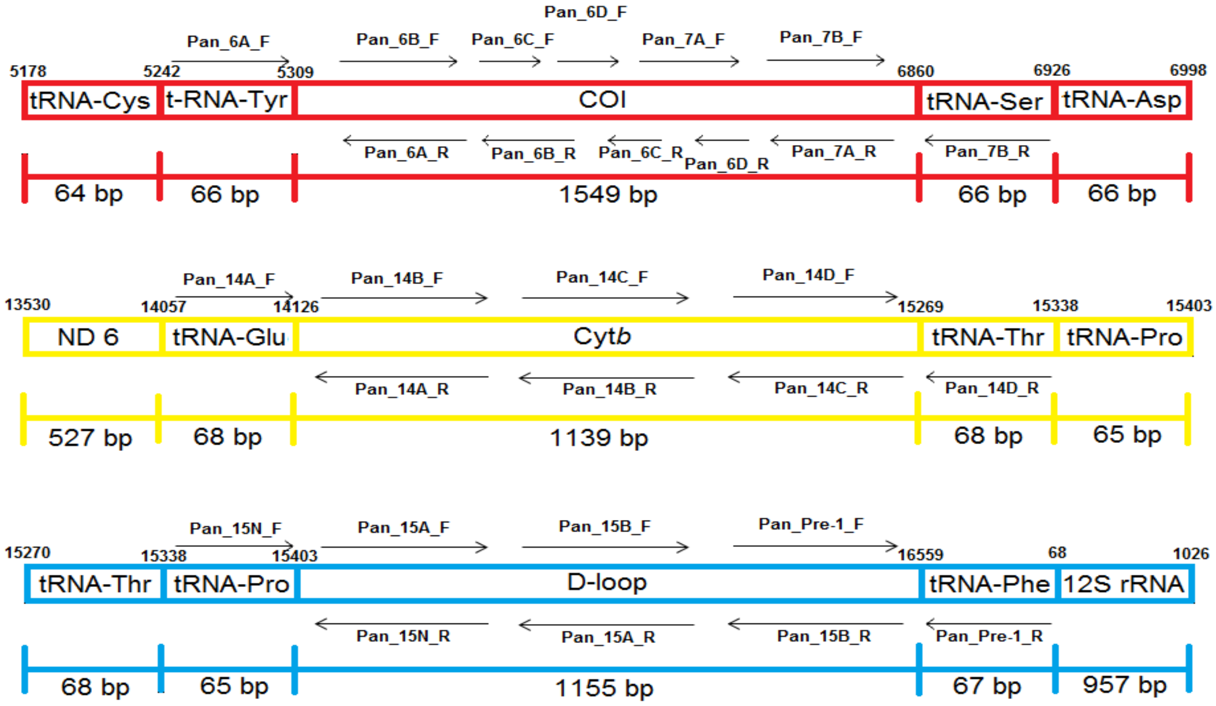

Figure 5.2: The respective position of primer sets and amplification size at the COI, Cyt*b* and D-loop gene regions used during the study. Numbering is according to the *S. temminckii's* whole mtDNA expenditure in Appendix A.

Species-specific markers for the three gene regions were designed using the whole mtDNA sequence of Temminck's ground pangolin (Appendix A). The gene regions were sequenced in both the forward and reverse directions using six primer sets for CO1, four primer sets for Cyt*b* and four primer sets for the D-loop region (Figure 5.2; Table 5.1).

Table 5.1: List of species-specific primer sequences used to sequence the COI, Cyt*b* and the D-loop mitochondrial gene regions of Temminck's ground pangolin. The expected fragment sizes of the sequences are indicated in base pairs (bp) and the annealing temperature used in the PCR is shown.

Gene Region	Primer Name	Primer Sequence	Fragment Size (bp)	Annealing Temperature (˚C)
COI	Pan_6A	F: 5'- TCA GCC ACC TTA CCT ATG TTC -3'	380-400	50
		R: 5'- GAT GAG ATA CCC GCT AAA TG -3'		
	Pan_6B	F: 5'- GCT GGA ACT GGC TGA ACT GTA -3'	400-440	53
		R: 5'- TTA CGC TGC CTC CAT GTA AGG -3'		
	Pan_6C	F: 5'- GCT GGA ACT GGC TGA ACT GT-3'	90-100	50
		R: 5'- TGA CTT ATA GCG GGA GGT -3'		
	Pan_6D	F: 5'- GGC TTT ATC GTT TGA GCA CAT C -3'	90-100	50
		R: 5'- AAG CCC TAA TGC TCA TAG TAG AG -3'		
	Pan_7A	F: 5'- CAC ACG AGC CTA CTT TAC ATC AGC -3'	300-330	50
		R: 5'- TGC CCG AAA GAC CAA GGA AGT GTT G -3'		
	Pan_7B	F: 5'- CAA CGA CAC ATG AGC AAA AG -3'	280-300	50
		R: 5'- CAT AGG TAT GAT ATT GGC TTG -3'		
Cyt*b*	Pan_14A	F: 5'- CCA TAA ATA GGA GAA GGC TTA GAA G -3'	230-250	50
		R: 5'- GTG TTC TGC TGT GTA GTG TAT TGC -3'		
	Pan_14B	F: 5'- CCC TCC AAC ATC TCA GCA T -3'	570-600	50
		R: 5'- AGG GGT CGG AAT ATC ATA GTG CGT T -3'		
	Pan_14C	F: 5'- CCC CTC CAA CAT CTC AGC AT -3'	130-150	50
		R: 5'- CCG TAA TAT AAG CCT CGT CC -3'		
	Pan_14D	F: 5'- AAC CCC CTA AGC ACA CCT CCC CAT A -3'	350-380	50
		R: 5'- GGG CTT TAG TCT CCT TCC TGA GTC -3'		
D-Loop	Pan_15N	F: 5'- AAG GAG ATT CTA ACC TCC CC -3'	330-360	50
		R: 5'- CCT TCA GTG GAG GTG ATA CG -3'		
	Pan_15A	F: 5'- CCA ACG GGC AAA TAC GCT ATG -3'	480-500	50
		R: 5'- GTC CTG CGA CCA TTG ACT GAA-3'		
	Pan_15B	F: 5'- GAC TGT GGG GTA GTT ATA GGA GAA -3'	450-480	50
		R: 5'- TTA G0+ AG CGG GCA GAA AAC TG -3'		
	Pan Pre-1	F: 5'- CAT CTT GTC AAA CCC CAA AAG C -3'	500-530	53
		R: 5'- GGC ACG AGA TTT ACC AAC CCA T -3'		

The Polymerase Chain Reaction was executed using 8.5 µl ddH$_2$O, 12.5 µl 2x DreamTaq^{TM} Mastermix, 1 µl (10 µM) of each primer pair and 2 µl DNA to a total PCR reaction volume of 25 µl. Thermal cycling was conducted as follows: initial denaturation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 30 sec, annealing at 50–53°C for 1 min, extension at

72°C for 45 sec, followed by final extension at 72°C for 7 min. PCR amplification was verified on 2% agarose gels, followed by PCR purification using exosap (Thermo Scientific, Lithuania). BigDye v3.1 Terminator Kit (Applied Biosystems, Foster City, CA) with the purified template DNA were used in a sequencing reaction and the ZR DNA Sequencing Clean-up^{TM} Kit (Zymo Research Corporation) to remove remnants from cycle sequencing. Capillary electrophoresis was performed on an ABI 3130 genetic analyser (Applied Biosystems, Foster City, CA).

5.3.3. BARCODING, HAPLOTYPE AND GENETIC VARIATION ANALYSIS

The Chromas programme embedded in MEGA v5.2 (Tamura *et al*., 2011) was used for viewing and editing of sequences and NCBI Basic Local Alignment Search Tool (BLAST) (www.ncbi.nm.nih.gov/blast) was used to carry out sequence blast searches in order to establish whether the sequences obtained were from the desired gene region. The MEGA v5.2 software was also used to assemble and trim each gene region. Sequences for each gene region were aligned using the online programme Mafft v7 (Standley, 2013). The programme jModeltest v2.1.3 (Darriba *et al.*, 2012) was used to determine the best fit model of sequence evolution for each gene region under the Akaike Information Criterion (AIC) (Akaike, 1974). The phylogenetic programme BEAST v1.7 (Drummond & Rambaut, 2007) was used to infer the estimated divergence times among the haplotypes for each gene region and a time-measured phylogeny was also obtained. The BEAST analysis was run over two million generations after which 25% of the trees were removed as burn-in. Tracer software (Drummond & Rambaut, 2007) was used to assess the trace files obtained in BEAST and to determine whether mixing was achieved as well as to select the suitable percentage burn-in. The divergence time used to calibrate the BEAST tree was the estimated divergence time calculated in Chapter four, between *P. tetradactyla* and *S. temminckii*. For gene regions to be combined in phylogenetic analyses, the same model of sequence evolution needs to be assumed for these regions since the mutation rates of different regions in the genome can differ in a single species due to nucleotide substitution per basepair per generation (Baer *et al*. 2007). The combined dataset, comprising the three mtDNA gene regions, was therefore subjected to partition analyses in PartitionFinder v1.1 (Lanfear *et al*., 2012), in order to determine which gene regions evolve under similar models and to select the best-fit partitioning schemes and models of molecular evolution. Partitions were defined according to gene regions and run using linked branch lengths and a greedy search, testing for the best fit models under the AIC. The haplotype data and genetic distance for each gene region were calculated using DNAsp v5 (Rozas & Rozas, 1995) and the median-joining haplotype networks were drawn with the programme NETWORK v4 (Bandelt *et al*., 1999). Haplotype and nucleotide diversity, pairwise comparison of genetic differentiation among populations (F_{ST}) and a study of hierarchical distribution of overall genetic diversity (Analysis of Molecular Variance AMOVA) were performed for each gene region using the software ARLEQUIN v3.5 (Excoffier & Lischer, 2010).

5.4. RESULTS

5.4.1. MITOCHONDRIAL DNA VARIATION AND HAPLOTYPE ANALYSIS

Table 5.2: List of models, conserved sites, variable sites, parsimony informative sites, gamma parameter, invariant sites, transition:transversion ratio and nucleotide frequencies for each gene region used during the analysis of the dataset.

	COI	**Cyt*b***	**D-loop**
Model	TIM2+G	TIM2+I	TPM2uf+G
Conserved Sites (C)	1141/1555	814/1135	854/1225
Variable Sites (V)	413/1555	321/1135	329/1225
Parsimony Informative (Pi)	120/1555	91/1135	83/1225
Gamma Parameter (G)	0.228	0	0.375
Invariant Sites (I)	0	0.42	0
Transition:Transversion Ratio (R)	3.64	2.15	1.54
Nucleotide Frequency (T)	32.6	28.3	31.1
Nucleotide Frequency (C)	22.6	27.6	21.4
Nucleotide Frequency (A)	28.0	31.7	32.4
Nucleotide Frequency (G)	16.8	12.5	15.1

The CO1 (1549 bp), Cyt*b* (1139 bp) and D-loop (1155 bp) gene regions were successfully sequenced for all 25 samples. The haplotype sequences for each gene region have been attached as Appendix B (CO1), Appendix C (Cyt*b*) and Appendix D (D-loop). The best-fit model of sequence evolution for each dataset, selected under AIC is presented in Table 5.2, along with the gamma (G) and invariant sites (I) parameters. The dataset characteristics, obtained for the datasets were also summarised in Table 5.2. The results from PartitionFinder showed that the three gene regions cannot be combined into one phylogenetic dataset as different models of evolution apply to each gene. The models obtained in PartitionFinder mirrored the models indicated in Table 5.2 for each gene region.

Table 5.3: Haplotype layout for COI gene region illustrating the number of haplotypes shared between individuals as the first value; the proportion of unique haplotypes as the second value; haplotype diversity and nucleotide diversity in the different populations observed during this study.

	Kalahari	Mpumalanga	Namibia	Zimbabwe	Mozambique	Museum
Haplotype 1	1/0.143	-	-	-	-	-
Haplotype 2	1/0.143	-	-	-	-	-
Haplotype 3	2/0.286	-	-	-	-	-
Haplotype 4	1/0.143	-	-	-	-	-
Haplotype 5	1/0.143	1/0.167	2/0.500	-	-	-
Haplotype 6	1/0.143	-	-	-	-	-
Haplotype 7	-	1/0.167	-	-	-	-
Haplotype 8	-	3/0.50	-	-	-	-
Haplotype 9	-	1/0.167	-	-	-	-
Haplotype 10	-	-	1/0.250	-	-	-
Haplotype 11	-	-	1/0.250	-	-	-
Haplotype 12	-	-	-	1/0.200	-	-
Haplotype 13	-	-	-	1/0.200	-	-
Haplotype 14	-	-	-	1/0.200	1/0.500	-
Haplotype 15	-	-	-	1/0.200	-	-
Haplotype 16	-	-	-	1/0.200	-	-
Haplotype 17	-	-	-	-	1/0.500	-
Haplotype 18	-	-	-	-	-	1/1
Proportion unique Haplotypes (%)	85.8	83.5	50.0	80.0	50.0	100.0
Haplotype Diversity (%)	95.2 (±9.6)	80.0 (±17.2)	83.3 (±22.2)	100.0 (±12.6)	100.0 (±50.0)	0.0
Nucleotide Diversity (%)	0.398	0.167	0.161	0.631	0.708	0.0

Table 5.4: Haplotype layout for Cyt*b* gene region illustrating the number of haplotypes haplotypes shared between individuals as the first value; the proportion of unique haplotypes as the second value; haplotype diversity and nucleotide diversity in the different populations observed during this study.

	Kalahari	**Mpumalanga**	**Namibia**	**Zimbabwe**	**Mozambique**	**Museum**
Haplotype 1	2/0.286	4/0.667	3/0.750	-	-	1/1
Haplotype 2	1/0.143	-	1/0.250	-	-	-
Haplotype 3	2/0.286	-	-	-	-	-
Haplotype 4	1/0.143	-	-	-	-	-
Haplotype 5	1/0.143	-	-	-	-	-
Haplotype 6	-	2/0.333	-	1/0.200	-	-
Haplotype 7	-	-	-	1/0.200	-	-
Haplotype 8	-	-	-	1/0.200	-	-
Haplotype 9	-	-	-	1/0.200	-	-
Haplotype 10	-	-	-	1/0.200	-	-
Haplotype 11	-	-	-	-	1/0.500	-
Haplotype 12	-	-	-	-	1/0.500	-
Proportion unique Haplotypes (%)	57.2	0.0	0.0	80.0	100.0	0.0
Haplotype Diversity (%)	90.5 (±10.3)	53.3 (±17.2)	50.0 (±26.5)	100.0 (±12.6)	100.0 (±50.0)	0.0
Nucleotide Diversity (%)	0.193	0.047	0.044	0.493	0.529	0.0

There were a total of 18 haplotypes observed for the CO1 gene region (Table 5.3), 12 haplotypes for Cyt*b* (Table 5.4) and 16 haplotypes for the D-loop region (Table 5.5). The haplotype and nucleotide diversity for all three gene regions are presented in Tables 5.3–5.5 and results indicate that the majority of the haplotypes found were unique for each geographic region. In the CO1 gene region two haplotypes were shared between sampling areas. Haplotype 5 was found in one individual from the Kalahari, one individual from Mpumalanga and two individuals from Namibia. Haplotype 14 was observed in one individual from Zimbabwe and one individual from Mozambique. For the Cyt*b* gene region, there were three haplotypes shared between sampling areas, namely haplotypes 1, 2 and 6. A similar pattern was observed for the D-loop region. There were three haplotypes (haplotypes 2, 4 and 14) shared between sampling areas, with the Kalahari, Mpumalanga and Namibia sharing one haplotype. The second haplotype is shared between Zimbabwe and Mozambique and the third is shared between the Kalahari and Mozambique. A total of 33 haplotypes were unique to individual populations.

Table 5.5: Haplotype layout for the D-loop gene region illustrating the number of haplotypes haplotypes shared between individuals as the first value; the proportion of unique haplotypes as the second value; haplotype diversity and nucleotide diversity in the different populations observed during this study.

	Kalahari	Mpumalanga	Namibia	Zimbabwe	Mozambique	Museum
Haplotype 1	1/0.143	-	-	-	-	-
Haplotype 2	2/0.286	2/0.333	1/0.250	-	-	-
Haplotype 3	2/0.286	-	-	-	-	-
Haplotype 4	1/0.143	-	-	-	1/0.500	-
Haplotype 5	1/0.143	-	-	-	-	-
Haplotype 6	-	2/0.333	-	-	-	-
Haplotype 7	-	1/0.167	-	-	-	-
Haplotype 8	-	1/0.167	-	-	-	-
Haplotype 9	-	-	1/0.250	-	-	-
Haplotype 10	-	-	1/0.250	-	-	-
Haplotype 11	-	-	1/0.250	-	-	-
Haplotype 12	-	-	-	1/0.200	-	-
Haplotype 13	-	-	-	1/0.200	-	-
Haplotype 14	-	-	-	2/0.400	1/0.500	-
Haplotype 15	-	-	-	1/0.200	-	-
Haplotype 16	-	-	-	-	-	1/1
Proportion unique Haplotypes (%)	57.2	66.8	75.0	60.0	0.0	100.0
Haplotype Diversity (%)	90.5 (±10.3)	86.7 (±12.9)	100.0 (±17.7)	90.0 (±16.1)	100.0 (±50.0)	0.0
Nucleotide Diversity (%)	0.483	0.149	0.559	0.929	0.946	0.0

None of the individuals were heteroplasmic and no nuclear homologues were presumably amplified since multiple bands were not observed. Across all three gene regions, none of the populations had exclusively private haplotypes. In general the haplotypes were only found in one or two individuals resulting in a high total haplotype diversity (H_T = 0.788–0.934), thus haplotype frequencies are potentially of limited use in determining population structure in pangolins. The nucleotide diversity for the three gene regions varied from 0.261–0.613%. Within individual geographic populations, there was considerable genetic variation as diversity varied from 0.161–0.708% for COI, 0.044–0.529% for Cyt*b* and 0.149–0.946% for the D-loop region (Tables 5.3–5.5).

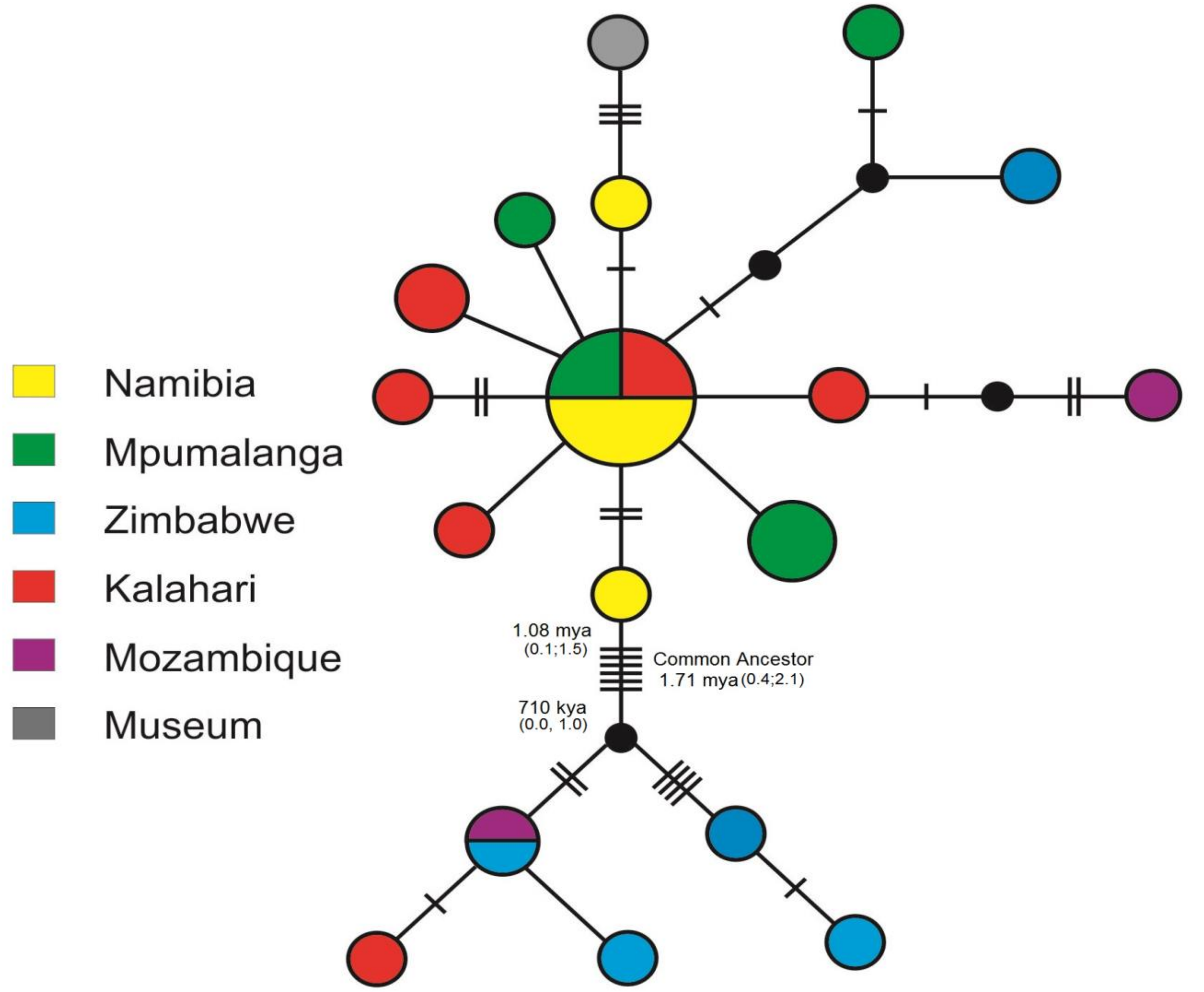

Figure 5.3: Haplotype network for the COI gene. Circles represent different haplotypes and the size of the circle indicates t0 he number of individuals that share that haplotype. Colours indicate the sampling locality of the haplotype. The black circles represent missing haplotypes and the black bars represent the amount of mutations the haplotype underwent. The common ancestor represents the time when the two groups (Group 1 and Group 2) shared the last common ancestor and the alternative dates represent the estimated time when the two groups split from the common ancestor. These molecular dates were calculated in BEAST with the respective 95% confidence intervals, for each date, presented in brackets and expressed in mya before the present.

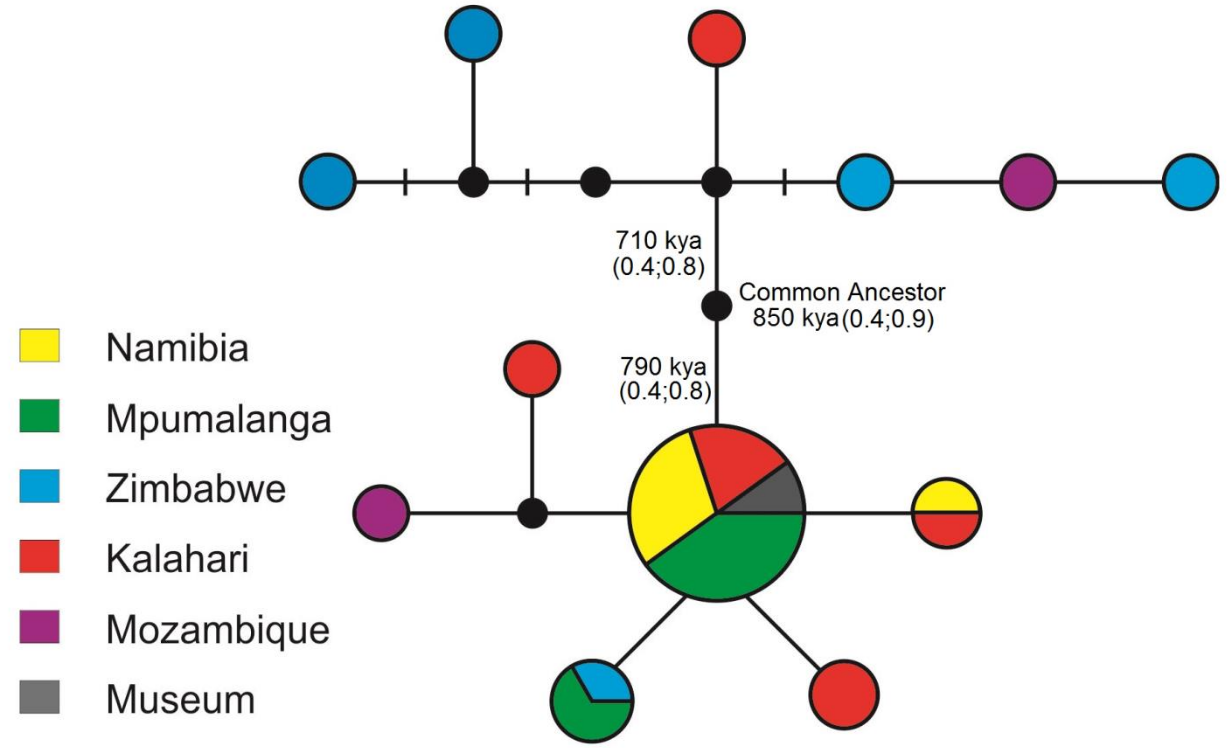

Figure 5.4: Haplotype network for the Cyt*b* gene. Circles represent different haplotypes and the size of the circle indicates the number of individuals that share that haplotype. Colours indicate the sampling locality of the haplotype. The black circles represent missing haplotypes and the black bars represent the amount of mutations the haplotype underwent. The common ancestor represents the time when the two groups (Group 1 and Group 2) shared the last common ancestor and the alternative dates represent the estimated time when the two groups split from the common ancestor. These molecular dates were calculated in BEAST with the respective 95% confidence intervals, for each date, presented in brackets and expressed in mya before the present.

Haplotype networks (Figures 5.3–5.5) indicate a clear split, for the majority of the samples, between the Zim/Moz region and Kal/Nam/MP samples. The results obtained from the BEAST analysis were extrapolated onto the branches of the haplotype networks to give an indication of divergence time between Group 1 (Zim/Moz) and Group 2 (Kal/Nam/MP). The BEAST results indicated that the two groups shared a common ancestor between 2.94 and 1.27 mya, across the three gene regions, depending on the mutation rate of each gene region (Figures 5.3–5.5).

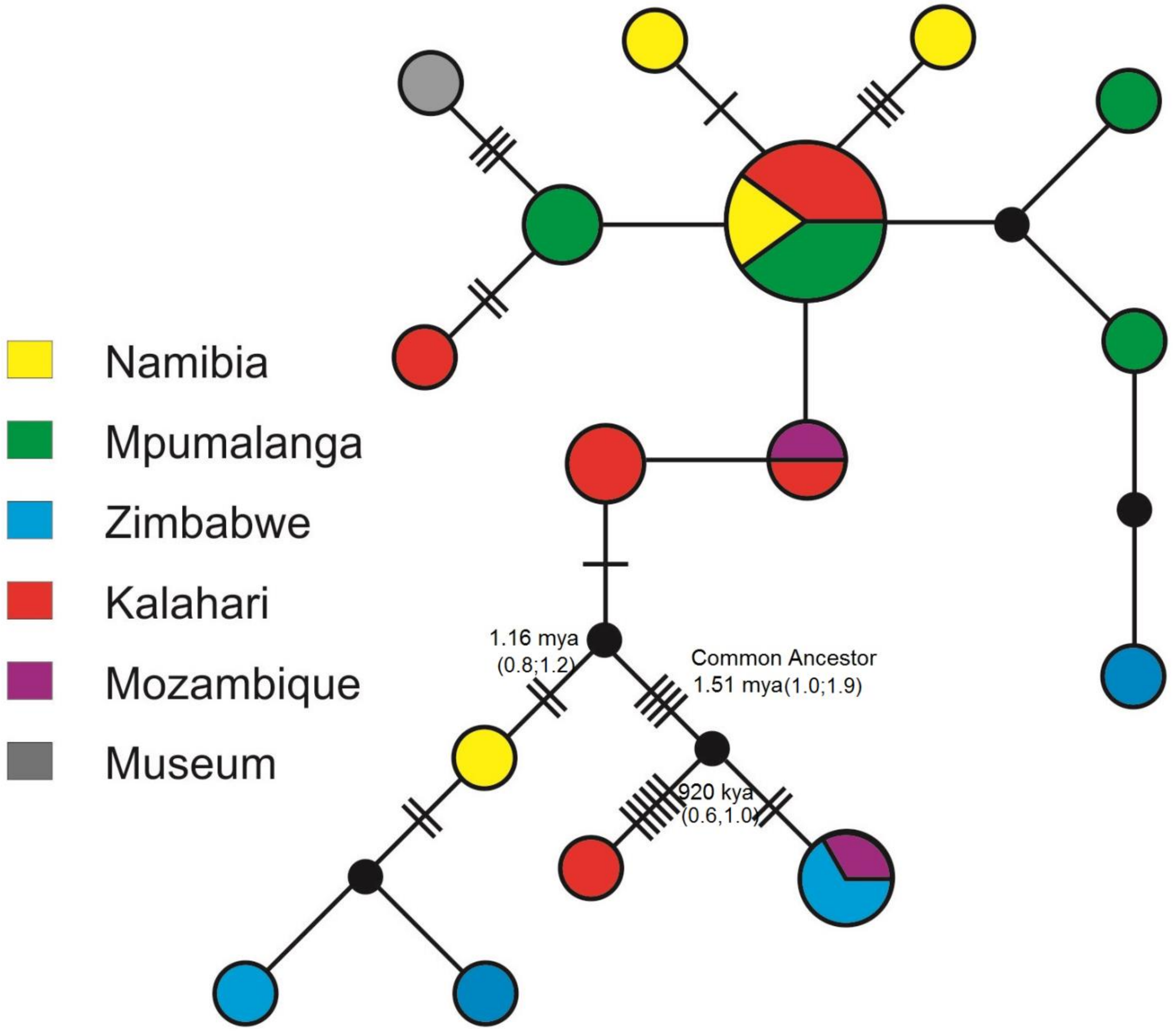

Figure 5.5: Haplotype network for the D-loop gene. Circles represent different haplotypes and the size of the circle indicates the number of individuals that share that haplotype. Colours indicate the sampling locality of the haplotype. The black circles represent missing haplotypes and the black bars represent the amount of mutations the haplotype underwent. The common ancestor represents the time when the two groups (Group 1 and Group 2) shared the last common ancestor and the alternative dates represent the estimated time when the two groups split from the common ancestor. These molecular dates were calculated in BEAST with the respective 95% confidence intervals, for each date, presented in brackets and expressed in mya before the present.

5.4.2. GENETIC DIFFERENTIATION (F_{ST}) AND GENETIC DISTANCE (G_{ST})

Table 5.6: Population pairwise distance (F_{ST}) for COI, Cyt*b* and D-loop gene region illustrating distance F_{ST} with significance level ($P<0.005$) below the horizontal and genetic distance (G_{ST}) above the horizontal. The bold values indicate the highest variation as well as the least variation between populations.

	Pairwise Fst (Significance level P<0.005); Genetic Distance (Gst)				
	Kalahari	**Mpumalanga**	**Namibia**	**Zimbabwe**	**Mozambique**
			CO1		
Kalahari	*	0.054	0.025	0.014	0.055
Mpumalanga	0.023 (0.234)	*	0.059	0.053	0.079
Namibia	**0.0 (0.940)**	0.071 (0.144)	*	0.044	0.059
Zimbabwe	0.38 (0.045)	0.543 (0.009)	0.447 (0.027)	*	0.0
Mozambique	0.252 (0.036)	**0.564 (0.054)**	0.483 (0.063)	**0.0 (0.721)**	*
			Cyt*b*		
Kalahari	*	0.058	0.035	0.026	0.063
Mpumalanga	0.061 (0.261)	*	0.0	0.100	0.149
Namibia	**0.0 (0.838)**	0.111 (0.234)	*	0.138	0.157
Zimbabwe	0.158 (0.027)	0.229 (0.027)	0.161 (0.081)	*	0.031
Mozambique	0.127 (0.207)	**0.439 (0.009)**	0.314 (0.108)	**0.0 (0.892)**	*
			D-loop		
Kalahari	*	0.011	0.0	0.052	0.039
Mpumalanga	0.021 (0.315)	*	0.0	0.062	0.065
Namibia	**0.0 (0.586)**	0.029 (0.306)	*	0.027	0.020
Zimbabwe	0.181 (0.072)	0.382 (0.0)	0.166 (0.144)	*	0.0
Mozambique	0.0 (0.378)	**0.409 (0.036)**	0.056 (0.324)	**0.0 (0.991)**	*

The highest level of differentiation and genetic distance (for CO1) was observed between Mpumalanga and Mozambique with an F_{ST} value of 0.564 (0.054) and G_{ST} value of 0.079 (Table 5.6). The lowest significant level of differentiation was observed between Zimbabwe–Mozambique and Kalahari–Namibia with F_{ST} 0.0 (0.940) / 0.0 (0.721) and G_{ST} 0.0 / 0.025 respectively. For the Cyt*b* region the largest differentiation was also observed between Mpumalanga and Mozambique with F_{ST} 0.439 (0.009) and G_{ST} 0.149 (Table 5.6). Zimbabwe–Mozambique and Kalahari–Namibia showed the lowest significant level of differentiation for Cyt*b* with F_{ST} 0.0 (0.838) / 0.0 (0.892) and G_{ST} 0.031 / 0.035 respectively. The same pattern was also observed in the D-loop region (Table 5.6) where the highest differentiation was observed between Mpumalanga and Mozambique with F_{ST} 0.409 (0.036) and G_{ST} 0.065. The regions with the lowest significant level observed were Zimbabwe–Mozambique and Kalahari–Namibia with F_{ST} 0.0 (0.586) / 0.0 (0.991) and G_{ST} 0.0 / 0.0 respectively.

Table 5.7: Genetic structure of all three gene regions using AMOVA to distinguish between and within different paired populations. The source of variation values displays the percentage of variation observed across all three gene regions. The Fixation Index (F_{CT}) with significant values $P<0.005$, represented in brackets, were also included for all three gene regions. Four different groupings were run with AMOVA. These include the five different populations (Kalahari, Namibia, Mpumalanga, Zimbabwe and Mozambique) as separate entities; West (Kal/Nam)–southeast (MP)–northeast (Zim/Moz) split; East (MP/Zim/Moz)–West (Kal/Nam) split and (Kal/MP/Nam)–(Zim/Moz) split.

	Source of variation								
	Among groups			**Among populations within groups**			**Within populations**		
	COI	**Cyt*b***	**D-loop**	**COI**	**Cyt*b***	**D-loop**	**COI**	**Cyt*b***	**D-loop**
Individual Groupings	31.92 -	13.12 -	13.40 -				68.08	86.88	86.60
West-southeast-northeast Split	40.75 [0.41 (0.06)]	19.47 [0.19 (0.12)]	22.09 [0.30 (0.10)]	0.0	0.0	0.0	63.35	84.0	83.34
East-West Split	0.0 [0.0 (0.49)]	0.86 [0.009 (0.40)]	0.0 [0.0 (0.60)]	34.81	12.53	18.19	69.02	86.61	88.76
(Kal/MP/ Nam)-(Zim/Moz) Split	50.12 [0.50 (0.10)]	27.75 [0.88 (0.10)]	30.25 [0.30 (0.10)]	0.0	0.0	4.64	52.17	75.64	74.38

A geographical approach and motivation was used to rationalise the four groupings chosen for the AMOVA analysis. The first analysis was performed with all five sampling areas treated as separate units (Table 5.7). Results showed that the highest percentage of genetic variation was observed within populations (68.08–86.88%) across the three gene regions. The lowest variation was observed among the geographic regions (13.12–31.92%). AMOVA was also performed with three different alternative groupings of the data (Table 5.7). The first run was based on an arrangement of Group 1 (Kal/Nam), Group 2 (MP) and Group 3 (Zim/Moz), representing a west-southeastern-northeastern split within southern Africa. The results indicate the same pattern across all three gene regions with the highest percentage of genetic variation being observed within populations (63.35–84.0%) and the lowest among groups (19.47–40.75%). The Fixation Index (F_{CT}) = 0.19–0.41 (0.06–0.12) across all three gene regions. The second analysis was performed between Group 1 (Kal/Nam) and Group 2 (MP/Moz/Zim) forming an east–west split within southern Africa. The same pattern was also observed in all three gene regions where the highest percentage of genetic variation occurred within populations (69.02–86.02%) and the lowest among groups (0.0–0.86%). The Fixation Index (F_{CT}) = 0.0–0.009 (0.4–0.6) across all three gene regions. The third AMOVA analysis was performed between Group 1 (Zim/Moz) and Group 2 (Kal/Nam/MP). The highest percentage genetic variation was observed within populations (52.17–75.64%) and the lowest among groups (27.75–50.12%). The Fixation Index (F_{CT}) = 0.30–0.88 (0.10) across all three gene regions. The Fixation Indexes (F_{CT}) provides stronger support for the AMOVA results since it validates the percentage variation found among groups. This is significant since we wanted to observe the differences among groups for each analysis in order to obtain the best geographical grouping for these populations as well as to test the hypothesis of the western and eastern split which we suspected.

5.5. DISCUSSION

Total haplotype diversity of Temminck’s ground pangolin (0.880 overall and site-specific values ranging from 0.788–0.934) is high and within the range reported for the Malayan pangolin, *M. javanica* (0.947; Wirdateti *et al.*, 2013). Furthermore, the haplotype diversity results obtained in this study are similar to those reported for antelope species, for example: Chinese water deer, *Hydropotes inermis inermis* (0.923; Hu *et al.*, 2006), Indian muntjac, *Muntiacus muntjak* (0.862; Wu & Fang, 2005), Kob, *Kobus kob* (0.72; Birungi & Arctander, 2000) and the African buffalo, *Syncerus caffer* (0.91; Simonsen *et al.*, 1998). Genetic diversity expressed as total nucleotide diversity in Temminck’s ground pangolin (0.429% overall and site-specific values ranging from 0.261–0.613%) is moderate and lower than values reported for the Malayan pangolin (2.5%*;* Wirdateti *et al.*, 2013) and also lower than estimates reported for antelope species, for example: Chinese water deer (1.318%), greater kudu, *Tragelaphus* *strepsiceros* (1%), impala, *Aepyceros melampus* (2.2%) and eland, *Taurotragus oryx* (0.5–3.6%), (Nersting & Arctander, 2001; Hu *et al*., 2006; Masembe *et al.*, 2006; Lorenzen *et al*., 2010).

This study found low levels of differentiation within the two main geographic units (Kal, Nam and MP) and (Zim and Moz), thus suggesting that there is still gene flow between Temminck's ground pangolins found within these regions. However, hierarchical analysis of the data suggests that two geographical units are present, namely Group 1 (which includes samples from Zimbabwe and Mozambique) and Group 2 (which includes samples from the Kalahari, Mpumalanga and Namibia), with a high genetic divergence between these regions. Across all three gene regions, AMOVA results indicate a substantial level of genetic differentiation (F_{CT} = 0.30–0.88; p = 0.10 overall and site-specific values ranging from 0.278–0.501) between Group 1 and Group 2. In addition, relatively little differentiation among populations within the same geographic regions was observed (F_{SC} site-specific values ranging from 0.0–0.046). These results are further supported by high levels of subdivision based on F_{ST} in all three gene regions between the two main geographic units. The highest levels of genetic distance were observed between Mpumalanga and Mozambique (F_{ST} = 0.439; p = 0.009) and between Mpumalanga and Zimbabwe (F_{ST} = 0.229; p = 0.027). However, the two main geographical units shared two out of 46 haplotypes (Tables 5.3–5.5). There are no obvious restrictions to gene flow within this species; thus, the observed differentiation between the two main geographical units may be due to a more recent phenomenon which could have restricted gene flow as a result of geographic distance, habitat fragmentation and/or behaviour or due to an ancient habitat-based separation.

It is a well-known phenomenon that individuals separated by geographic distance often display large genetic distance as observed in various African Bovids (Templeton & Georgiadis, 1996). Ground pangolins however are known to travel long distances as Van Aarde *et al.* (1990) reported on a young male Temminck's ground pangolin fitted with a Very High Frequency (VHF) transmitter at Olifantshoek in the Northern Cape Province being recaptured four months later in the Northwest Province, 300 km from the release site. The inferred dispersal rate of 2.5 km a day is within the range of 2–7 km a day recorded in a previous study (Pietersen, 2013), although the possibility that this was a human-assisted dispersal event cannot be entirely ruled out. If this was a *bona fide* dispersal event this could indicate that juveniles may disperse between the sampling localities, since the species' distributional range is continuous, thus maintaining gene flow between regions and sampling areas. Further evidence of possible dispersal between sampling localities was found by Pietersen (2013), where tracked animals dispersed minimum distances of 32–81 km in 20 days before contact was lost or the transmitters removed. Thus it is unlikely that isolation-by-distance itself is resulting in differentiation between the two main genetic sub-populations identified during this study. However, morphological and habitat differences between these two sub-populations of pangolin have been observed, particularly with regards to the average size of individuals (Pietersen, 2013). In addition, portions of this species' range have been transformed due to agricultural practices leading to unsuitable habitat (APWG, 2013). In addition territorial behaviour and natal philopatry may play a role in genetic differentiation (Sugg *et al.,* 1996; Piertney *et al.,* 1998). Although the Temminck's ground pangolins are known as solitary animals with large territories and strong home range fidelity, little is currently known with regards to their behaviour. Thus a combination of habitat differences, substantial geographic distance and intrinsic factors may lead to a degree of gene isolation and genetic drift establishing this divergence between the two sub-populations. A stepping stone model of migration could be suggested since the dispersal habitat of Temminck's ground pangolin is considered to be one enormous overlapping distribution across southern Africa (Figure 5.1). The stepping-stone model suggests that migration is restricted to adjacent sub-populations as would be the circumstance with the pangolins where the populations are not isolated and neighbouring populations (especially juveniles) are able to disperse and exchange genetic material, thus maintaining a high genetic gene pool (Kimura & Weiss, 1964). However, additional sampling in the intervening regions is required to further investigate this hypothesis. Lastly, several species namely African bovids such as impala and Greater kudu as well as giraffes are genetically differentiated into South-eastern and South-western southern African populations (Arctander *et al*., 1999; Nersting & Arctander, 2001; Hassanin *et al*., 2007), which may be attributed to the Mega Kalahari Sand Sea forming a barrier to gene flow (Stokes *et al*., 1998). Thus, these results may reflect an ancient habitat-based separation which coincides with the Mega Kalahari Sand Sea phenomenon, since we estimate that the Zim/Moz split occurred between 920 and 710 kya and the Kal/Nam/MP split between 1.16 mya and 790 kya.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

This study was the first to investigate the population genetics of Temminck's ground pangolin in southern Africa. This study forms part of a larger project to determine the genetic structure and phylogenetic patterns of pangolins in Africa. The original aims of this study were met as follows:

6.1. OPTIMIZATION OF SCALE SAMPLES

Non-invasive sampling techniques have become more popular for usage in genetic studies as it has low impact on animals compared to invasive sampling. Furthermore, the pangolin's defence mechanism consists of rolling into a ball when threatened, thus making the collection of blood samples difficult whereas scale clippings are easily obtainable. Pangolin scales consist of keratin, the same composition as human nails, which makes it an attractive alternative of sampling from pangolins. However, extracting DNA from scale samples is challenging as the DNA is usually degraded or fragmented. The results obtained during this study demonstrated that obtaining good quality sequencing data from scale samples is possible by using a variety of DNA isolation protocols such as the QIAamp® DNA Investigator Kit which assists in obtaining higher quality DNA. Various PCR enhancing protocols such as addition of BSA and glycerol can also assist to increase the quality of the extracted DNA and the success of the amplification process during PCR. High quality sequences were obtained following usage of the various PCR enhancing techniques and ranged between 100–500 bp, depending on the respected primer amplification length for each region. The success rate obtained from the sequences, after using all the extraction protocols and PCR enhancing techniques, amounted to successful amplification of approximately 89% of the samples used during this study.

6.2. WHOLE MITOCHONDRIAL DNA ANALYSIS AND PHYLOGENETICS

The study presented here indicates that Temminck's ground pangolin mtDNA genome is 16,559 bp in length. These results were generated by using the primer walking method along with ten cross-species markers generated from the black-bellied pangolin (*Phataginus tetradactyla*) as well as 48 species-specific markers in order to fill the remaining gaps in the genome. A comparison between the whole mtDNA genomes of Temminck's ground pangolin (*Smutsia temminckii*), the black-bellied pangolin (*P. tetradactyla*) and the Chinese pangolin (*Manis pentadactyla*) indicated that these three pangolin species differ considerably on a genetic basis. Various differences as well as similarities in the different gene regions were noted. These were observed between the three pangolin species as well as between the two African species respectively. Genetic differences would be expected since the Chinese pangolin is found in Asia, the black-bellied pangolin is an arboreal species from Africa and Temminck's ground pangolin is a ground-dwelling species from Africa. This differentiation was also confirmed during the phylogenetic analysis where the separation between the species was dated. The results further indicated that pangolins are more closely related to the order Carnivora and Erinaceomorpha compared to the order Xenarthra, as would be expected. In addition, results confirm a Laurasian origin for the pangolins, estimated at 87 mya, from where they gradually descended into Africa while some individuals ascended further into Asia. We also suspect that the arboreal species evolved from the ground-dwelling pangolins in Africa since the fossils found in Africa are more closely related to the giant ground pangolin than to the arboreal pangolin species. No fossils have been located for the arboreal species and limited information is available on the other pangolin species. In addition, full mtDNA analysis has yet to be undertaken for the giant ground pangolin (*Smutsia gigantea*) and white-bellied pangolin (*Phataginus tricuspis*).

6.3. PHYLOGEOGRAPHY, GENETIC STRUCTURE AND DIVERSITY

Results from this study indicated that there is a genetic separation between Group 1 (Zimbabwe/Mozambique) and Group 2 (Kalahari/Namibia/Mpumalanga) of Temminck's ground pangolin populations in southern Africa. The AMOVA results indicated a significant level of genetic differentiation suggesting the presence of two geographical units with little differentiation observed between populations in the same geographic unit. These observations were further supported by high levels of genetic distance (F_{ST}) observed between

Mpumalanga and Mozambique (F_{ST} = 0.439 (0.009)) and between Mpumalanga and Zimbabwe (F_{ST} = 0.229 (0.027)). This separation between the two geographical units could either be caused by an ancestral division such as the formation of the Mega Kalahari Sand Sea during the Pleistocene era or a more recent barrier of unsuitable habitat. The same pattern of separation was observed across all three mtDNA gene regions, with differentiation as well as haplotype network analysis indicating similar results. However, these findings should be regarded as preliminary in the absence of data from nuclear DNA analysis. Such data could verify whether the southern populations of the species may contain smaller units for conservation, whether as ESUs or sub-species. Since this study forms part of a larger project, no conservation plans can be implemented presently with reverence to ESUs since there is still a lot of information absent regarding these species, both genetically as well as morphologically. In addition, larger sample sets from a wider distribution of the species within its range is required.

6.4. RECOMMENDATIONS

In concordance with the above mentioned observation noted during the mtDNA analysis, additional nuclear DNA markers could be developed to assist in obtaining more conclusive analysis on Temminck’s ground pangolin populations along with the mtDNA results. Single Nucleotide Polymorphisms and development of species-specific microsatellite markers can contribute by giving a more precise indication of population structure in these species. Developing species-specific microsatellite markers would also have better success in amplifying *S. temminckii* DNA than the cross-species markers since it would be developed from the same species thus containing similar DNA compositions located in each cell. A previous study performed by De Beer (2013) indicated that cross-species markers have limited success in amplifying Temminck’s ground pangolin genomic DNA, thus justifying the necessity for alternative methods. Larger sample sets covering a wider distribution range, of these animals, will also support more accurate results obtained from both mtDNA and nuclear DNA analysis. This in turn will contribute to a better understanding of the population structure of Temminck’s ground pangolin and as a further result, the rest of the African pangolin species.

SUMMARY

Temminck's ground pangolin, *S. temminckii*, is currently listed as *Vulnerable* on the IUCN Red Data List. However, their numbers are decreasing due to illegal hunting for bush meat and over-harvesting for traditional use in Africa. Pangolins are also exported to Asia as a delicacy and for use in traditional medicine. Currently, the greatest threat to ground pangolins in southern Africa is electrocution by electric fences on game farms. This project consisted of two parts. The first was to sequence the whole mtDNA genome of Temminck's ground pangolin to identify gene regions and to determine the evolutionary relationship of the order Pholidota. Results generated using the primer walking method, indicated that the whole mtDNA of Temminck's ground pangolin is 16,559 bp in length. The phylogenetic analysis shows that the order Pholidota form a sister grouping with the order Carnivora rather than with the order Xenarthra as would be expected. Data suggested a Laurasian origin approximately 87 mya and possible migration into Africa during the Paleocene era around 55 mya. The second part of the study was conducted in order to determine the phylogeography of Temminck's ground pangolins in southern Africa. Twenty five samples were collected from four countries, namely Namibia, Zimbabwe, Mozambique and South Africa (Mpumalanga and the Northern Cape Provinces). The results obtained indicated a high level of genetic variation within populations and only a few individuals displayed private haplotypes, which resulted in an increase in haplotype diversity. Samples from Zimbabwe and Mozambique (Group 1) clustered together while samples from the Northern Cape and Mpumalanga Provinces of South Africa grouped with samples from Namibia (Group 2), suggesting either an ancestral or recent split between Groups 1 and 2. The BEAST analysis indicated that the two groups shared a recent common ancestor between 2.94 and 1.27 mya across the three gene regions. In addition, it was estimated that the Zimbabwe/Mozambique split occurred between 920 and 710 kya and the Kalahari/Namibia/Mpumalanga split between 1.16 mya and 790 kya. This pattern corresponds to the Mega Kalahari Sand Sea forming a barrier between individuals and populations around that time. This study is the first molecular analysis based on the mitochondrial DNA genome of Temminck's ground pangolin in southern Africa and it provides an insight into the species' population genetics across its range in southern Africa. However, additional research into the order Pholidota throughout Africa can assist in better understanding of genetic variation within African pangolin species and populations. Furthermore, such studies will also support the conservation of genetic variation within species and contribute to identifying evolutionary distinct populations to assist in developing effective conservation management plans for the different species of the order Pholidota.

KEYWORDS: mitochondrial DNA, phylogeny, phylogeography, *Smutsia temminckii*, Temminck's ground pangolin

OPSOMMING

Die huidige status van Temminck se grond itermagog, *S. temminckii*, is gelys as *Kwesbaar* op die Internasionale Unie vir die Bewaring van Natuurlewe (IUCN) se Rooi Data Lys. Nietemin, toon bevolkings ‘n klaarblyklike afname in getalle as gevolg van onwettige jagtery vir wildsvleis, sowel as groot stropings aanvraag vir tradisionêle gebruik in Afrika. Itermagoë word ook na Asië uitgevoer waar hulle beskou word as ‘n lekkerny en ook gebruik word in tradisionele medisyne. Die grootste gevaar vir itermagoë in Suider-Afrika is huidiglik doodskokking deur elektrisiteitsdrade op wildsplase. Hierdie projek het bestaan uit twee dele. Die eerste deel het behels om deur middle van volgordebepaling die volledige mitokondriale deoxyribonukleïensuur (mtDNS) genoom van Temminck se grond itermagog vas te stel, om die verskillende geen areas te identifiseer en evolusionêre verwantskappe van die orde Pholidota te bepaal. Resultate wat met behulp van die “primer walking” metode gegenereer is, het getoon dat die volledige mtDNS van Temminck se grond itermagog 16,559 bp lank is. Die filogenetiese analise het getoon dat die orde Pholidota ‘n sister groep vorm met die orde Carnivora eerder as groepeer met die orde Xenarthra soos verwag. Gevolglik toon die data ook ‘n Laurasiëse oorsprong, ongeveer 87 miljoen jaar gelede, sowel as moontlike migrasie na Afrika gedurende die Paleocene tydperk ongeveer 55 miljoen jaar gelede. Die tweede deel van die studie het gehandel oor die filo-geografie van Temminck se grond itermagog in Suider-Afrika. Vyf-en-twintig individue, afkomstig van vier lande, is bestudeer. Die lande het betaan uit Namibië, Mosambiek, Zimbabwe en Suid-Afrika (Mpumalanga- en Noord-Kaap Provinsies). Die resultate toon dat daar ‘n hoë vlak van genetiese divergensie tussen bevolkings is en net ‘n paar individue het unieke haplotiepes getoon wat gelei het tot verhoging in haplotiep diversiteit. Die resultate het verder getoon dat die individue van Zimbabwe en Mosambiek (Groep 1) saam groepeer terwyl individue van Noord-Kaap Provinsie en Mpumalanga Provinsie saam groepeer met individue van Namibië (Groep 2). Bogenoemde groeperings kan te wyte wees aan ‘n voorouer of moderne skeiding tussen die twee groepe. Die BEAST analise het getoon dat die twee groepe ‘n gemeenskaplike voorouer gedeel het tussen 2.94 en 1.27 miljoen jaar gelede oor die drie geen areas. Die resultate het verder getoon dat die Zimbabwe/Mosambiek skeiding tussen 920 en 710 duisend jaar gelede ontstaan het en die Kalahari/Mpumalanga/Namibië skeiding tussen 1.16

miljoen jaar gelede en 790 duisend jaar gelede oor die drie geen areas. Bogenoemde patroon is te wyte aan die Groot Kalahari Sand-See wat ‘n versperring tussen individue en bevolkings van daardie tyd gevorm het. Hierdie studie is die eerste molekulêre analise gebaseer op die mtDNS genoom van Temminck se grond itermagog in Suider-Afrika en dit veskaf ‘n beter insig tot die spesie se bevolkingsgenetika regoor hul verspreidings gebied in Suider-Afrika. Additionele navorsing van die orde Pholidota regoor Afrika is nietemin nodig ten einde ‘n beter begrip van die genetiese variasie in itermagog spesies en bevolkings te verkry. Die resultate dra ook by tot die bewaring van genetiese diversiteit in identifisering van unieke bevolkings, wat verder sal bydra tot die ontwikkeling van effektiewe bewaringsbestuursplanne vir al die spesies in die orde Pholidota.

SLEUTELTERME: filogenie, filo-geografie, mitokondriale DNS, *Smutsia temminckii*, Temminck se grond ietermagog

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APPENDIX A

WHOLE MITOCHONDRIAL DNA SEQUENCE OF TEMMINCK'S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*)

APPENDIX A

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LOCUS       Smutsia_temminckii     16559 bp    DNA     circular DNA
FEATURES             Location/Qualifiers
     Source          1..16559
                     /organism="Smutsia temminckii"
                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
     tRNA            1..68
                     /label=tRNA-Phe
     rRNA            69..1026
                     /label="12S ribosomal RNA"
     tRNA            1027..1092
                     /label=tRNA-Val
     rRNA            1093..2646
                     /label="16S ribosomal RNARNA"
     tRNA            2647..2721
                     /label=tRNA-Leu
     CDS             2726..3681
                     /gene=ND1
                     /note="TAA stop codon is completed by the
                      addition of 3' A
                     residues to the mRNA"
                     /codon_start=1
                     /transl_except=(pos:3680..3681,aa:TERM)
                     /transl_table=2
                     /product="NADH dehydrogenase subunit 1"

/translation=MSIINLLLTIIPILLAVAFLTLVERKVLGYMQLRKGPNIVGPWGLL

QPIADAMKLFTKEPLRPLTSSIMMFIMAPAMALTLALTMWMPLPMPHPLININLGVLFM

LAMSSLAVYSILWSGWASNSKYALIGAIRAVAQTISYEVTLAIILLSVLLMNGSFTLPT

LIITQEKLWLLVPTWPLAMMWFISTLAETNRAPFDLTEGESELVSGFNVEYAAGPFALF

FLAEYANIIMMNSLSTIIFLGATHSPYSPNLYTANLVTKVLILTMTFLWIRASYPRFRY

DQLMHLLWKNFLPLTLAMCMWHISVPIATSSIPPQT
     Gene            2726..3681
                     /label=ND1
     tRNA            3682..3750
                     /label=tRNA-Ile
     tRNA            complement(3748..3819)
                     /label=tRNA-Gln
     tRNA            3821..3889
                     /label=tRNA-Met
     CDS             3890..4931
                     /gene=ND2
                     /note="TAA stop codon is completed by the
                      addition of 3' A
                     residues to the mRNA"
                     /codon_start=1
                     /transl_except=(pos:4931,aa:TERM)
                     /transl_table=2
                     /product="NADH dehydrogenase subunit 2"

/translation=MNPLALILIMSTVFFSTTLVLISSNWLTIWVGFEMNMLAMIPILMK
```

```
KFNPRATEAATKYFLIQATASMLLMAAITMNFIHSGEWTVTKMCNTIPSTIITMAMAMK

LGMSPFHFWMPEVTQGIPLSSGMLLLTWQKIAPISVLYQIMPTINLNLMLAMALLSILI

GGWGGLNQTQLRKIMAYSSIAHLGWMMAIMLYNPNIAILNLLIYILMTLSTFMMLHHNS

TTTTLSLSHLSNKMPLMTIILLLILMSMGGLPPLSGFMPKWMIIQELTKNDMMLLPTLM

AMMALLNLYFYLRLTYSTTLTMFPTPNNMKMTWQFQHNKHMKLVSPLIILSTMILPLTP
                     IMSILI
     Gene            3890..4931
                     /label=ND2
     tRNA            4932..4999
                     /label=tRNA-Trp
     tRNA            complement(5002..5070)
                     /label=tRNA-Ala
     tRNA            complement(5072..5144)
                     /label=tRNA-Asn
     tRNA            complement(5178..5242)
                     /label=tRNA-Cys
     tRNA            complement(5243..5309)
                     /label=tRNA-Tyr
     CDS             5311..6860
                     /gene=COX1
                     /codon_start=1
                     /transl_table=2
                     /product="cytochrome c oxidase subunit I"

/translation=MFINRWLFSTNHKDIGTLYLIFGAWAGMVGTALSLLIRAELGQPGT

LLGDDQIYNVVVTAHAFVMIFFMVMPIMIGGFGNWLVPLMIGAPDMAFPRMNNMSFWLL

PPSFLLLLASSMVEAGAGTGWTVYPPLAGNLAHAGASVDLTIFSLHLAGISSILGAINF

ITTIINMKPPAMSQYQTPLFVWSVLVTAVLLLLSLPVLAAGITMLLTDRNLNTTFFDPA

GGGDPILYQHLFWFFGHPEVYILILPGFGMISHIVTYYSGKKEPFGYMGMVWAMMSIGF

LGFIVWAHHMFTVGMDVDTRAYFTSATMIIAIPTGVKVFSWLATLHGGSVKWAPALLWA

LGFIFLFTVGGLTGIVLANSSLDIVLHDTYYVVAHFHYVLSMGAVFAIMGGFVHWFPLF

SGYTLNDTWAKVHFTIMFVGVNMTFFPQHFLGLSGMPRRYSDYPDAYTLWNTVSSMGSF

ISLTAVMLMVFMIWEAFASKREILMVELTDTNLEWLHGCPPPYHTFEEPAFVNPMKQ
     Gene            5311..6860
                     /label=COX1
     tRNA            complement(6856..6926)
                     /label=tRNA-Ser
     tRNA            6932..6998
                     /label=tRNA-Asp
     CDS             6999..7682
                     /gene=COX2
                     /codon_start=1
                     /transl_table=2
```

```
                     /product="cytochrome c oxidase subunit II"

/translation=MAHPFQLGFQDATSPIMEELLHFHDHTLMIVFLISSLVLYIISLML

TTKLTHTNTMDAQEVETIWTVLPAIILILIALPSLRILYMMDEINNPSLTVKTMGHQWY

WSYEYTDYEDLTFDSYMIPTQDLKPGELRLLEVDNRLVLPMEMTIRMLISSEDVLHSWA

VPSLGLKTDAIPGRLNQTTLMSHRPGLFFGQCSEICGSNHSFMPIVIEMVPLKTFENWS
                     LSML*
     Gene            6999..7682
                     /label=COX2
     tRNA            7685..7751
                     /label=tRNA-Lys
     CDS             7754..7954
                     /gene=ATP8
                     /codon_start=1
                     /transl_table=2
                     /product="ATP synthase F0 subunit 8"

/translation=MPQLDTSTWFIMITSMFLTLFILLQLKISKYNYPHNAEIATKLDYK

ISTPWMKKWTKIYLPLSLPQ*
     Gene            7754..7954
                     /label=ATP8
     CDS             7915..8595
                     /gene=ATP6
                     /codon_start=1
                     /transl_table=2
                     /product="ATP synthase F0 subunit 6"

/translation=MNENLFASFLTPMMMGIPIVTIIIMFPIIMFPSPKRLINNRIESMQ

QWLLQQTTKQMMSIHSYKGQTWTLMLMSLIIFIASTNLLGLLPYSFTATTQLSMNLGMA

VPLWAATVITGFRHKTKMSLAHLLPQGTPTPLIPVLVIIETISLLIQPMALAVRLTANI

TAGHLLMHLIGSATLALMSISLTVATPTFIILVLLTILEFAVALIQAYVFTLLISLYLH

DNT*
     Gene            7915..8595
                     /label=ATP6
     CDS             8595..9378
                     /gene=COX3
                     /note="TAA stop codon is completed by the
                      addition of 3' A
                     residues to the mRNA"
                     /codon_start=1
                     /transl_except=(pos:9378,aa:TERM)
                     /product="cytochrome c oxidase subunit III"

/translation=MNHQTHSYHMVNPSPWPLTGALSALLMTSGLAMWFHFNSMILLSLG

LITNFLTMYQWWRDIVRESTFQGHHTMTVQKGLRYGMILFIISEIFFFIGFFWAFYHSS

LAPTLELGGCWPPTGINPLNPMEVPLLNTSVLLASGVSITWAHHSLMEGNRNHMIQALL
```

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ITISLGLYFTLLQISEYYEAPFTISDGVYGSTFFMATGFHGLHVIIGSSFLLVCFMRQL

KFHFTSKHHFGFEAAAWYWHFVDVVWLFLYVSIYWWGS
     Gene            8595..9378
                     /label=COX3
     tRNA            9379..9447
                     /label=tRNA-Gly
     CDS             9448..9793
                     /gene=ND3
                     /note="TAA stop codon is completed by the
                      addition of 3' A
                     residues to the mRNA"
                     /codon_start=1
                     /transl_except=(pos:9793,aa:TERM)
                     /transl_table=2
                     /product="NADH dehydrogenase subunit 3"

/translation=MNLVMMMMINVLLASLLMLIAFWLPQLYIYSEKVTPYECGFDPMGS

ARLPFSMKFFLIAITFLLFDLEIVLLLPLPWASQTNQLNIMLITALLLLLLLAISLAYE
                     WTQKGLEWAE
     Gene            9448..9793
                     /label=ND3
     tRNA            9794..9861
                     /label=tRNA-Arg
     CDS             9862..10158
                     /gene=ND4L
                     /codon_start=1
                     /transl_table=2
                     /product="NADH dehydrogenase subunit 4L"

/translation=MPSIHINIMLAFVISLAGMLMYRSHLMSSLLCLEGMMLSLFTMIAI

TSLNINFTLASMAPIILLVFAACEAALGLSLLVLVSNSYGTDYVQNLNLLRC
     Gene            9862..10158
                     /label=ND4L
     CDS             10152..11529
                     /gene=ND4
                     /note="TAA stop codon is completed by the
                      addition of 3' A
                     residues to the mRNA"
                     /codon_start=1
                     /transl_except=(pos:11529,aa:TERM)
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12121 CTCCGCTAAC AATTTATTTC AACTCTTTAT CGGCTGAGAA GGAGTAGGCA TTATATCCTT
12181 CCTACTAATT GGTTGATGAT ACGGTCGAAC AGACGCAAAC ACAGCTGCCA TACAAGCAAT
12241 CCTATACAAC CGTATTGGCG ACATTGGATT TCTCCTAGCT ATAGCATGAT TCTTCACAAA
12301 ATCAAACTCA TGGGACCTCC AACAAATTTT TATTATTAAC TATGAAAACA CAAACATTCC
12361 CTTAGCCGGC CTACTACTAG CCGCAACAGG AAAATCCGCT CAATTCGGAC TACATCCCTG
12421 ATTACCTTCA GCAATAGAAG GTCCCACCCC AGTATCAGCA TTACTTCACT CCAGTACAAT
12481 AGTAGTAGCA GGAGTATTCC TCCTGATCCG ATTCTACCCT TTAATAGAAA ACAACAAAAC
12541 AATACAAACA ATAACCCTAT GCTCAGGAGC CATTACAACC CTATTTACAG CTATCTGCGC
12601 TTTAACCCAA AATGATATTA AAAAAATCGT AGCATTTTCA ACCTCAAGCC AACTAGGATT
12661 AATAATAGTT ACTATCGGTA TTAACCAACC ACACTTAGCC TTTATTCATA TCTGTACTCA
12721 CGCCTTCTTC AAAGCCATAT TATTTATGTG CTCAGGGTCA ATTATCCACA ACCTAGAAAA
12781 CGAACAAGAT ATCCGCAAAA TAGGCGGCTT ATTCCACGCC CTGCCCCTCA CAACCTCCGC
12841 CCTTATCATC GGAAGCCTGG CCCTAACAGG CATGCCTTTC CTTACAGGAT TTTACTCCAA
12901 AGACCTCATT ATCGAATCTA TCAACATGTC TTATACAAAC GCCTGAGCCC TCTCCATCAC
12961 TCTACTAGCT ACTTCCCTAA CAGCTGCATA CAGTACCCGC ATAGTATTTC TCGTATTAAC
13021 AGGCCAACCC CGATTTACTG CCCTAAATGC TATTAATGAA AATAATCCAG CACTAACTAA
13081 TTCACTTAAA CGTCTAATAC TAGGAAGCAT CTTCGCCGGA TTCTTGTTAC TTAACTATAT
13141 TCCCCCAACA ACCACCCCCC TAATAACCAT ACCATACTAC CTAAAACTCA CCACTCTATT
13201 AGTCACAGTA CTAGGATTTA TTGTAGCATT AGAACTATGT AACCTATCTA CAAATCTTCA
13261 ACATAATGAA CCCTCAAACA CATTTAAATT TTCAAACTCA TTAGGCTACT TCCCAACAAT
13321 CATCCATCGA ACCGCCCCCA TATCTACATT AGTAATAAGC CAAAAACTAG CATCCCTAAT
13381 AATAGACTTA ACCTGACTAG AAAAAATCTT ACCAAAAGCC ACTTCACACT TCCACTTACA
13441 GGCATCTTCC TTAACATCAA ATCACAAAGG CCTAATTAAA CTATACTTCC TATCATTCAT
13501 AATGACAATT ACTCTAGCCC TGCTAACATC TAATTACCTC GAGTAATTTC CATAACAATA
13561 ATTACACACA TTAACAAAGA TCATCCAGTA ACAACAATAA ACCAAACTCC ATAGCTGTAC
13621 AAAGCAGAAA CCCCCATAAT ATCTCCACTT AAAATTCCAA GTCCCCCAGT ATCATAAACC
13681 ATTCAATCAC CTTTACTACT AAAATCTAAC ACTACTCCCA TTTCTTCGCC CTTAATTATA
13741 AAATAAACCA ACACTAACTC TATTGCCGAC CCAATAACAA ATATGCCTAA TACAACTACA
13801 TTAGAAACCC AAGCCTCAGG ATATTCCTCA ATGGCTATAG CAGATGTATA TCCAAAAACA
13861 ACCAACATAC CCCCAAGATA GATTAAAAAA ACTATTAAAC CTAAAAAAGA CCCACCAGAA
13921 CACACCACAA TTCCACATCC CACACCTCCC GCCACAATCA AACTAAATCC CCCATAAATA
13981 GGAGAAGGCT TAGAAGAAAC CCCTAAGAAA CTAATTACAA AAACAACACT CAAGACAAAC
14041 ACAACATATA TTGCCATTAT TCTTACATGG ACTCTAACCA TGGCCTATGA CATGAAAAAT
14101 CATCGTTGTA ACTAAACTAC AAGAACTTAA TGACAAATAT ACGAAAATCT CACCCCCTAT
14161 TTAAAATCAT CAGTAATTCT TTTATTGACC TCCCAACCCC CTCCAACATC TCAGCATGAT
14221 GAAACTTTGG CTCTCTATTA GGAATATGCT TAATCCTACA AATTATAACA GGACTATTCT
14281 TAGCAATACA CTACACAGCA GACACTACAA CCGCATTCTC ATCAATTACT CATATCTGTC
14341 GAGATGTGAA CTATGGCTGA ATTATCCGTT ACATACATGC CAATGGCGCA TCAATATTCT
14401 TCATCTGCTT ATTTATCCAT ATTGGACGAG GCTTATATTA CGGATCCTTC ACCTACAAAG
14461 AAACATGAAA CATCGGAATT ATCCTCTTAT TTACAGTTAT AGCAACAGCC TTCGTAGGGT
14521 ATGTCCTACC ATGAGGACAA ATATCATTCT GAGGGGCTAC AGTAATTACT AACCTCTTAT
14581 CAGCAATCCC CTATATCGGT CCAGACCTGG TAGAATGAAT CTGAGGGGGC TTCTCAGTTG
14641 ACAAAGCAAC TCTAACTCGA TTCTTCGCAT TTCACTTCAT CTTCCCCTTT ATTATTACAG
14701 GCCTAGCAAT AGTCCACCTA CTATTCCTTC ACGAAACAGG ATCCAACAAT CCAATAGGCC
14761 TTATCTCCAA TTCAGACAAA ATCCCATTTC ACCCGTACTA CACGATCAAA GACATCCTAG
14821 GTCTATTCAT TATATCTATA GCCCTAATAA CTCTAGTACT ATTTTTCCCC GACCTTCTAG
14881 GAGACCCAGA CAATTACACA CCAGCTAACC CCCTAAGCAC ACCTCCCCAT ATTAAGCCAG
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    14941 AATGATATTT TCTATTTGCA TACGCAATCC TACGATCAAT TCCCAACAAA CTAGGAGGTG
    15001 TACTAGCCTT AGCCATATCC ATCCTAATCT TAGCCATTAT CCCCTCATTA CAAATATCAA
    15061 AACAACGCAC TATGATATTC CGACCCCTAA GCCAAATCAT ATTCTGACTC CTAGTAGCAA
    15121 ACCTCCTCGT ACTTACCTGA ATTGGAGGAC AACCCGTGGA ACACCCATTC ATCCTAATCG
    15181 GCCAAGTAGC CTCAATCTCA TACTTTACCC TCATCCTAGT AATATTACCA ATTGCAAGCA
    15241 CTATTGAAAA TAAACTTATA AAATGAAGAG TCTCTGTAGT ATATCTAATA CACTGGCCTT
    15301 GTAAACCAAA AAAGGAGATT CTAACCCTCC CCGAGACTCA GGAAGGAGAC TAAAGCCCCT
    15361 CCATCAACAC CCAAAGCTGA CATTCTCTAT AAACTACTTC CTGAAAAATT TTATACAACA
    15421 TAAAACCCCA CCAACGGGCA AATACGCTAT GTATAATCGT GCATTAATGG CTGGCCCCAT
    15481 GAATATAAGC AAGTACTAAT AAATCATTAT AGTACATAGG ACATATTATG TATAATCGTG
    15541 CATTAATGAT ATGCCCCATG CATATAAGCA GGTACTATCA ATTATTACAG TACATAGGAC
    15601 ATATTATGTA TAATCGTGCA TTACTGATAT ACCCCATGCA TATAAGCAAG TACTATTAAC
    15661 CATTAAAGTA CATAGGACAT ATATATTATT AATCGTGCAT ACCACATCCA AGTCAAATCA
    15721 TTTCCAGTCA ACATGCGTAT CACCACCTCT GAAGGCCGCT TAATCACCAT GCCGCGTGAA
    15781 ATCATCAACC CGCCCACAAC GTGTCCCTCT TCTCGCTCCG GGCCCATATA GACTGTGGGG
    15841 TAGTTATAGG AGAACTATAC CTGGCATCTG GTTCTTACTT CATGTTCATT CGTCTATAGT
    15901 CGCTCACTCG TTCCTCTTAA ATAAGACATC TCGATGGATT AGTTACTAAT CAGCCCATGC
    15961 CGATGCATAA CTGTGGTGTC ATGCCCTTGG TATTTTTTAA TTTTAGGGTG CGGGGGTTCC
    16021 ACTAGGCGGA GCCTCAGAGC GGAGGACATT GAATGTGGTC AAGACATATA TTTAACTATT
    16081 ATTGGTCGTA CATATACTAC TTTCAGGTGT TATTCAGTCA ATGGTTACGG GACATATAAA
    16141 ATTTTACTAC AACATTTCAA GTCAACAATT TTTCCATCAT ACATATAAAT TTATTTTTAC
    16201 GTAGCTTAGT ATTCAAACCC CCCCTTACCC CCCCAATCTT ACATGTACTA AACATCTTGT
    16261 CAAACCCCAA AAGCAAGAAT ACATACACTG TAAGTGCTAC AAAAAACATA AAGTATTACG
    16321 TAAGCATTCA CGTAAGCATT TGGCTCAAAC CTTGTTACAC AGTTTTCTGC CTGCTCTAAA
    16381 AACACTTTAT TGTTTGGACA TATATGATCT GGAAATCTTA TAGTAATTAT AGTATTAGAA
    16441 TATTTTATTA ATGTAACATA GAGAATATAA TTTTTAAACC TACGCAAAAT ATTTACGCAT
    16501 TTATTTATTC TTATAGGCGT GCATAATGCA AACTATCTGC TCCCTACATG CAAATTAAA
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APPENDIX B

HAPLOTYPE MITOCHONDRIAL DNA SEQUENCES FOR CYTOCHROME *C* OXIDASE I (CO1) OF TEMMINCK'S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*)

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                      10         20         30         40         50         60         70         80         90
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01  ATGTTCATTA ATCGTTGATT ATTTTCAACT AACCACAAAG ATATCGGTAC ATTATACCTT ATCTTTGGCG CTTGAGCTGG GATAGTAGGA
Haplotype_02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06  .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
Haplotype_07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09  .......... .......... .......... .......... .......... G......... .......... .......... ..........
Haplotype_10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12  .......... .......... .......... ..T....... .......... .......... .......... .......... ..........
Haplotype_13  .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
Haplotype_14  .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
Haplotype_15  .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
Haplotype_16  .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
Haplotype_17  .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
Haplotype_18  .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
                     100        110        120        130        140        150        160        170        180
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01  ACCGCTTTAA GCCTCCTAAT CCGAGCTGAA TTAGGCCAAC CCGGAACCTT ATTAGGTGAT GACCAGATTT ATAACGTAGT TGTAACTGCA
Haplotype_02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06  .......... .......... .......... .......... .......... .......... .....A.... .C........ ..........
Haplotype_07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13  .......... .......... .......... .......... .......... .......... ..T..A.... .......... ..........
Haplotype_14  .......... .......... .......... .......... .......... .......... .....A.... .......... ..........
Haplotype_15  .......... .......... .......... .......... .......... .......... ..T..A.... .......... ..........
Haplotype_16  .......... .......... .......... .......... .......... .......... .....A.... ....G..... ..........
Haplotype_17  .......... .......... .......... .......... .......... .......... .....A.... .C........ ..........
Haplotype_18  .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                           190        200        210        220        230        240        250        260        270
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01       CACGCATTCG TAATAATTTT CTTTATAGTT ATACCTGTTA TAATTGGAGG TTTTGGAAAT TGGCTAGTGC CTTTGATAAT TGGTGCTCCC
Haplotype_02       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03       .......... .......... .......... ......A... .......... .......... .......... .......... ..........
Haplotype_04       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_07       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13       .......... .......C.. ...C...... .......... .......... .......... .......... .......... ..........
Haplotype_14       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_15       .......... .......C.. ...C...... .......... .......... .......... .......... .......... ..........
Haplotype_16       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_17       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18       .......... .......... .......... .......... .......... .......... .......... .......... ..........
                           280        290        300        310        320        330        340        350        360
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01       GACATGGCAT TTCCCCGTAT AAATAATATA AGTTTTTGAC TTCTCCCACC TTCATTTCTT CTTCTCCTAG CATCTTCTAT AGTAGAAGCT
Haplotype_02       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_07       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_14       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_15       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_16       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_17       .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18       .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                         370        380        390        400        410        420        430        440        450
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01     GGGGCTGGAA CTGGCTGAAC TGTATATCCA CCCTTAGCTG GTAACTTAGC ACATGCGGGA GCCTCAGTAG ACTTAACTAT TTTTTCCCTT
Haplotype_02     .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03     .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04     .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05     .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06     .......... .......... .......... .......... .......... .......... ........G. .......... ..........
Haplotype_07     .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08     .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09     .......... .......... .......... .......... .......... .......... ........G. .......... ..........
Haplotype_10     .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11     .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12     .......... .......... .......... .......... .......... .......... ........G. .......... ..........
Haplotype_13     .......... .......... .......... ........C. .......... .......... ........G. .......... ..........
Haplotype_14     .......... .......... .......... .......... .......... .......... ........G. .......... ..........
Haplotype_15     .......... .......... .......... ........C. .......... .......... ........G. .......... ..........
Haplotype_16     .......... .......... .......... .......... .......... .......... ........G. .......... ..........
Haplotype_17     .......... .......... .......... .......... .......... .......... ........G. .......... ..........
Haplotype_18     .......... .......... .......... .......... .......... .......... .......... .......... ..........
                         460        470        480        490        500        510        520        530        540
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01     CATTTAGCGG GTATTTCATC AATTTTAGGA GCTATTAACT TTATCACAAC TATCATCAAC ATAAAACCTC CCGCTATAAG TCAATATCAA
Haplotype_02     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06     .......... ....C..... .......... .......... .......... .......... .......... .T........ .........G
Haplotype_07     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13     .....G.... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_14     .......... ....C..... .......... .......... .......... .......... .......... .T........ .........G
Haplotype_15     .....G.... ....C..... .......... .......... .......... .......... .......... .T........ ..........
Haplotype_16     .......... ....C..... .......... .......... .......... .......... .......... .T........ .........G
Haplotype_17     .......... ....C..... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18     .......... ....C..... .......... .......... .......... .......... .......... .T........ ..........
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                       550        560        570        580        590        600        610        620        630
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01    ACTCCACTAT TTGTATGATC AGTACTAGTC ACAGCTGTAT TGCTCCTACT ATCCCTTCCA GTACTAGCTG CTGGAATTAC AATGCTTCTA
Haplotype_02    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04    .......... .......... G......... .......... .......... .......... .......... .......... ..........
Haplotype_05    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06    .......... .......... .......... .......... .......... .......... ..G....... .......... ..........
Haplotype_07    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10    .......... .......... .......... .......... .......... .......... ..G....... .......... ..........
Haplotype_11    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13    .......... .......... .......... .......... .......... .......... ..G....... .......... ..........
Haplotype_14    .......... .......... .......... .......... .......... .......... ..G....... .......... ..........
Haplotype_15    .......... .......... .......... .......... .......... .......... ..G....... .......... ..........
Haplotype_16    .......... .......... .......... .......... .......... .......... ..G....... .......... ..........
Haplotype_17    .......... .......... G......... .......... .......... .......... .......... .......... ..........
Haplotype_18    .......... .......... .......... .......... .......... .......... ..G....... .......... ..........
                       640        650        660        670        680        690        700        710        720
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01    ACTGACCGAA ATTTAAATAC AACCTTTTTT GACCCTGCTG GAGGGGGGGA CCCTATTCTA TATCAACATC TATTTTGATT CTTTGGTCAT
Haplotype_02    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_03    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_04    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_05    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_06    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_07    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_08    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_09    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_10    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_11    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_12    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_13    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_14    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_15    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_16    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_17    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype_18    .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
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                      730        740        750        760        770        780        790        800        810
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01  CCTGAGGTGT ACATTCTAAT TCTTCCTGGT TTTGGTATAA TCTCTCACAT CGTAACCTAT TATTCAGGAA AAAAAGAACC ATTTGGGTAC
Haplotype_02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06  .......... .......... .......... .......... .......... .......... .......... .......... ......A...
Haplotype_07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10  .......... .T........ .......... .......... .......... .......... .......... .......... ..........
Haplotype_11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13  .......... .T........ .......... .......... .......... .......... .......... .......... ..........
Haplotype_14  .......... .......... .......... .......... .......... .......... .......... .......... ......A...
Haplotype_15  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_16  .......... .......... .......... .......... .......... .......... .......... .......... ......A...
Haplotype_17  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18  .......... .T........ .......... .......... .......... .......... .......... .......... ..........
                      820        830        840        850        860        870        880        890        900
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01  ATAGGTATAG TCTGAGCAAT AATATCAATC GGTTTCCTAG GCTTTATCGT TTGAGCACAT CACATATTCA CAGTAGGTAT AGATGTTGAC
Haplotype_02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08  .......... .......... .......... .......... .......... ......G... .......... .......... ..........
Haplotype_09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_14  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_15  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_16  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_17  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18  .......... .......... .......... ....C.A... .......... .......... .......... .......... ..........
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                     910        920        930        940        950        960        970        980        990
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01  ACACGAGCCT ACTTTACATC AGCTACCATA ATTATTGCAA TTCCAACAGG CGTAAAGGTA TTTAGCTGAC TAGCTACCTT ACATGGAGGC
Haplotype_02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_14  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_15  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_16  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_17  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18  ..................................................................................................
                    1000       1010       1020       1030       1040       1050       1060       1070       1080
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01  AGCGTAAAAT GAGCTCCTGC TCTACTATGA GCATTAGGCT TTATTTTCCT ATTTACAGTG GGGGGACTAA CTGGAATTGT TCTAGCAAAC
Haplotype_02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06  .......... .......... .......... ........T. .......... .......... .......... ........A. ..........
Haplotype_07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11  .......... .......... .......... ........T. .......... .......... .......... .......... ..........
Haplotype_12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13  .......... .......... .......... ........T. .......... .......... .......... .......... ..........
Haplotype_14  .......... .......... .......... ........T. .......... .......... .......... .......... ..........
Haplotype_15  .......... .......... .......... ........T. .......... .......... .......... .......... ..........
Haplotype_16  .......... .......... .......... ........T. .......... .......... .......... .......... ..........
Haplotype_17  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18  .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                              1090       1100       1110       1120       1130       1140       1150       1160       1170
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01 TCATCATTAG ATATTGTTCT CCACGATACT TATTATGTAG TAGCCCATTT TCACTATGTT CTCTCTATAG GCGCAGTCTT CGCTATTATA
Haplotype_02 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06 .......... .......... .......... ..C....... .......... .......... .......... .......... ..........
Haplotype_07 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11 .......... .......... .......... ..C....... .......... .......... .......... .......... ..........
Haplotype_12 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13 .......... .......... .......... ..C....... .......... .......... .......... .......... ..........
Haplotype_14 .......... .......... .......... ..C....... .......... .......... .......... .......... ..........
Haplotype_15 .......... .......... .......... ..C....... .......... .......... .......... .......... ..........
Haplotype_16 .......... .......... .......... ..C....... .......... .......... .......... .......... ..........
Haplotype_17 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18 .......... .......... .......... .......... .......... .......... .......... .......... ..........
                              1180       1190       1200       1210       1220       1230       1240       1250       1260
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01 GGGGGATTTG TACACTGATT CCCACTATTC TCAGGATACA CTCTCAACGA CACATGAGCA AAAGTTCACT TTACTATTAT ATTCGTAGGA
Haplotype_02 .......... .......... .......... .......... ....T..... .......... .......... .......... ..........
Haplotype_03 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06 ..A....... .......... .......... .......... .......T.. .......... .......... .......... ..........
Haplotype_07 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09 .......... .......... .......... .......... .......... .......... .......... .......C.. ..........
Haplotype_10 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11 .......... .......... .......... .......... .......T.. .......... .......... .......... ..........
Haplotype_12 .......... .......... .......... .......... .......... .......... .......... .......C.. ..........
Haplotype_13 .......... .......... .......... .......... .......T.. .......... .......... .......... ..........
Haplotype_14 ..A....... .......... .......... .......... .......T.. .......... .......... .......... ..........
Haplotype_15 .......... .......... .......... .......... .......T.. .......... .......... .......... ..........
Haplotype_16 ..A....... .......... .......... .......... .......T.. .......... .......... .......... ..........
Haplotype_17 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18 .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                      1270      1280      1290      1300      1310      1320      1330      1340      1350
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01  GTAAATATGA CCTTTTTTCC CCAACACTTC CTTGGTCTTT CGGGCATACC ACGACGATAC TCCGATTATC CAGATGCTTA TACATTATGA
Haplotype_02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06  .......... ......C... .......... .......... .......... .......... .......... .......... ..........
Haplotype_07  .......... .......... .......... .......... .......... .......... .......... .......... ....C.....
Haplotype_08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09  .......... ......C... .......... .......... .A........ .......... .......... .......... ..........
Haplotype_10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12  .......... ......C... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13  .......... ......C... .......... .......... .......... .......... .......... .......... ..........
Haplotype_14  .......... ......C... .......... .......... .......... .......... .......... .......... ..........
Haplotype_15  .......... ......C... .......... .......... .......... .......... .......... .......... ..........
Haplotype_16  .......... ......C... .......... .......... .......... .......... .......... .......... ..........
Haplotype_17  .......... ......C... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18  .......... .......... .......... .......... .......... .......... .......... .......... ..........
                      1360      1370      1380      1390      1400      1410      1420      1430      1440
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01  AATACTGTAT CCTCTATAGG ATCCTTCATT TCATTAACCG CTGTAATACT GATAGTCTTT ATAATTTGAG AAGCATTTGC CTCTAAACGA
Haplotype_02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_13  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_14  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_15  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_16  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_17  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype_18  .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                        1450      1460      1470      1480      1490      1500      1510      1520      1530
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype_01   GAAATTCTCA TAGTAGAACT AACTGACACA AACCTCGAAT GACTACATGG CTGTCCTCCA CCCTATCACA CATTCGAAGA ACCTGCCTTT
Haplotype_02   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_03   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_04   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_05   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_06   .......... .......... .........G .......... .......... .......... .....C.... .......... ..........
Haplotype_07   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_08   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_09   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_10   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_11   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_12   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_13   .......... .......... .........G .......... .......... .......... .....C.... .......... ..........
Haplotype_14   .......... .......... .........G .......... .......... .......... .....C.... .......... ..........
Haplotype_15   .......... .......... .........G .......... .......... .......... .....C.... .......... ..........
Haplotype_16   .......... .......... .........G .......... .......... .......... .....C.... .......... ..........
Haplotype_17   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
Haplotype_18   .......... .......... .......... .......... .......... .......... .....C.... .......... ..........

                        1540      1550
                ....|....| ....|....| ....
Haplotype_01   GTCAACCCAA TAAAACAAGA AAGG
Haplotype_02   .......... .......... ....
Haplotype_03   .......... .......... ....
Haplotype_04   .......... .......... ....
Haplotype_05   .......... .......... ....
Haplotype_06   .......... .......... ....
Haplotype_07   .......... .......... ....
Haplotype_08   .......... .......... ....
Haplotype_09   .......... .......... ....
Haplotype_10   .......... .......... ....
Haplotype_11   .......... .......... ....
Haplotype_12   .......... .......... ....
Haplotype_13   .......... .......... ....
Haplotype_14   .......... .......... ....
Haplotype_15   .......... .......... ....
Haplotype_16   .......... .......... ....
Haplotype_17   .......... .......... ....
Haplotype_18   .......... .......... ....
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APPENDIX C

HAPLOTYPE MITOCHONDRIAL DNA SEQUENCES FOR CYTOCHROME *B* (CYT*B*) OF TEMMINCK’S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*)

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                      10         20         30         40         50         60         70         80         90
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  ATGACAAATA TACGAAAATC TCACCCCCTA TTTAAAATCA TCAGTAATTC TTTTATTGAC CTCCCAACCC CCTCCAACAT CTCAGCATGA
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
Haplotype 07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .........A .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     100        110        120        130        140        150        160        170        180
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  TGAAACTTTG GCTCTCTATT AGGAATATGC TTAATCCTAC AAATTATAAC AGGACTATTC TTAGCAATAC ACTACACAGC AGACACTACA
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  ..G....... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  ..G....... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  ..G....... .......... .......... .......... .......... .......... .......... .......... ..........
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                     190        200        210        220        230        240        250        260        270
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  ACCGCATTCT CATCAATTAC TCATATCTGT CGAGATGTGA ACTATGGCTG AATTATCCGT TACATACATG CCAATGGCGC ATCAATATTC
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .......... .......... C......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     280        290        300        310        320        330        340        350        360
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  TTCATCTGCT TATTTATCCA TATTGGACGA GGCTTATATT ACGGATCCTT CACCTACAAA GAAACATGAA ACATCGGAAT TATCCTCTTA
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                     370        380        390        400        410        420        430        440        450
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  TTTACAGTTA TAGCAACAGC CTTCGTAGGG TATGTCCTAC CATGAGGACA AATATCATTC TGAGGGGCTA CAGTAATCAC TAACCTCTTA
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......T.. ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     460        470        480        490        500        510        520        530        540
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  TCAGCAATCC CCTATATCGG TCCAGACCTG GTAGAATGAA TCTGAGGGGG CTTCTCAGTT GACAAAGCAA CTCTAACTCG ATTCTTCGCA
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......A.. .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                     550        560        570        580        590        600        610        620        630
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  TTTCACTTCA TCTTCCCCTT TATTATTACA GGCCTAGCAA TAGTCCACCT ACTATTCCTT CACGAAACAG GATCCAACAA TCCAATAGGC
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  ..C....... .......... .......... .......... .......... .......... .......... .......T.. ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  ..C....... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......T.. ..........
Haplotype 09  ..C....... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  ..C....... .......... .......... .......... .......... .......... .......... .......T.. ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  ..C....... .......... .......... .......... .......... .......... .......... .......T.. ..........

                     640        650        660        670        680        690        700        710        720
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  CTTATCTCCA ATTCAGACAA AATCCCATTT CACCCGTACT ACACGATCAA AGACATCCTA GGTCTATTCA TTATATCTAT AGCCCTAATA
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                     730        740        750        760        770        780        790        800        810
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  ACTCTAGTAC TATTTTTCCC CGACCTTCTA GGAGACCCAG ACAATTACAC ACCAGCTAAC CCCCTAAGCA CACCTCCCCA TATTAAGCCA
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... ....T..... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... ....T..... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     820        830        840        850        860        870        880        890        900
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  GAATGATATT TTCTATTTGC ATACGCAATC CTACGATCAA TTCCCAACAA ACTAGGAGGT GTACTAGCCT TAGCCATATC CATCCTAATC
Haplotype 02  .......... .......... ....A..... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .....G.... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .....G.... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  ...G...... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                      910        920        930        940        950        960        970        980        990
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  TTAGCCATTA TCCCCTCATT ACAAATATCA AAACAACGCA CTATGATATT CCGACCCCTA AGCCAAATCA TATTCTGACT CCTAGTAGCA
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... ....A..... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .....A.... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......A.. .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  .......... .......... .......... .......A.. .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... A......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......A.. .......... .......... .......... .......... ..........

                     1000       1010       1020       1030       1040       1050       1060       1070       1080
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  AACCTCCTCG TACTTACCTG AATTGGAGGA CAACCCGTGG AACACCCATT CATCCTAATC GGCCAAGTAG CCTCAATCTC ATACTTTACC
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .......... .......... .......... .......... .......... .......... ......A... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... ......A... .......... ..........
Haplotype 10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                         1090      1100      1110      1120      1130
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|
Haplotype 01   CTCATCCTAG TAATATTACC AATTGCAAGC ACTATTGAAA ATAAACTTAT AAAAT
Haplotype 02   .......... .......... .......... .......... .......... .....
Haplotype 03   .......... .......... .......... .......... .......... .....
Haplotype 04   .......... .......... .......... .......... .......... .....
Haplotype 05   .......... .......... .......... .......... .......... .....
Haplotype 06   .......... .......... .......... .......... .......... .....
Haplotype 07   .......... .......... .......... .......... .......... .....
Haplotype 08   .......... .......... .......... .......... .......... .....
Haplotype 09   .......... .......... .......... .......... .......... .....
Haplotype 10   .......... .......... .......... .......... .......... .....
Haplotype 11   .......... .......... .......... .......... .......... .....
Haplotype 12   .......... .......... .......... .......... .......... .....
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APPENDIX D

HAPLOTYPE MITOCHONDRIAL DNA SEQUENCES FOR CONTROL REGION (D-LOOP) OF TEMMINCK'S GROUND PANGOLIN (*SMUTSIA TEMMINCKII*)

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                    10         20         30         40         50         60         70         80         90
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01 CTACTTCCTG AAAAATTTTA TACAACATAA AACCCCACCA ACGGGCAAAT ACGCTATGTA TAATCGTGCA TTAATGGCTG GCCCCATGAA
Haplotype 02 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 13 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 14 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 15 .......... .......... .......... .G........ .......... .......... .......... .......... ..........
Haplotype 16 .......... .......... .......... .......... .......... .......... .......... .......... ..........

                   100        110        120        130        140        150        160        170        180
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01 TATAAGCAAG TACTAATAAA TCATTATAGT ACATAGGACA TATTATGTAT AATCGTGCAT TAATGATATG CCCCATGCAT ATAAGCAGGT
Haplotype 02 .......... .......... C......... .......... .......... .......... .......... .......... ..........
Haplotype 03 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04 .......... .......... C......... .......... .......... .......... .......... .......... ..........
Haplotype 05 .......... .......... C.....C... .......... .......... .......... .......... .......... .......A..
Haplotype 06 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07 .......... .......... C......... .......... .......... .......... .......... .......... ....A.....
Haplotype 08 .......... .......... C......... .......... .......... .......... .......... .......... ....A.....
Haplotype 09 .......... .......... C......... .......... .......... .......... .......... .......... ..........
Haplotype 10 .......... .......... C......... .......... .......... .......... .......... .......... ..........
Haplotype 11 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12 .......... .......... C......... .......... .......... .......... .......... .......... ..........
Haplotype 13 .......... .......... .......... .......... .......... .......... .....G.... .......... ..........
Haplotype 14 .......... .......... .......... .......... .......... .......... .....G.... .......... ..........
Haplotype 15 .......... ......C... .......... .......... .......... .......... .......... .......... .......A..
Haplotype 16 .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                        190       200       210       220       230       240       250       260       270
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01    ACTATTAATT ATTACAGTAC ATAAGACATA TTATGTATAA TCGTGCATTA CTGATATACC CCATGCATAT AAGCAAGTAC TATTAACCAT
Haplotype 02    .....C.... .......... ...G...... .......... .......... .......... .......... .......... ..........
Haplotype 03    .....C.... .......... ...G...... .......... .......... .......... .......... .......... ..........
Haplotype 04    .....C.... .......... ...G...... .......... .......... .......... .......... .......... ..........
Haplotype 05    .......... .......... ...G...... .......... .......... .......... T......... .......... ......T...
Haplotype 06    .....C.... .......... ...G...... .......... .......... .......... .......... .......... ..........
Haplotype 07    .....C.... .......... ...G...... .......... .......... .......... .......... .......... ..........
Haplotype 08    .....C.... .......... ...G...... .......... .......... .......... .......... .......... ..........
Haplotype 09    .....C.... .......... ...G...... .......... .......... .......... .......... .......... ..........
Haplotype 10    .....C.... .......... ...G...... .......... .......... .......... .......... .......... ..........
Haplotype 11    .....C.... .......... ...G...... .......... .......... .....T.C.. T......... .......... ..........
Haplotype 12    .....C.... .......... ...G...... .......... .......... .......... .......... .......... ..........
Haplotype 13    .......... .......... ...G...... .......... .......... .......... .......... .....G.... ......T...
Haplotype 14    .......... .......... ...G...... .......... .......... .......... .......... .....G.... ......T...
Haplotype 15    .....C.... .......... ...G...... .......... .......... .....T.C.. T......... .......... ..........
Haplotype 16    .....C.... .......... ...G...... .......... .......... .......... .......... .......... ..........

                        280       290       300       310       320       330       340       350       360
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01    TAAAGTACAT AGGACATACA TATTATTAAT CGTGCATACC ACATCCAAGT CAAATCATTT CCAGTCAACA TGCGTATCAC CACCTCTGAA
Haplotype 02    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03    .......... ........T. .......... .......... .......... .......... .......... .......... ..........
Haplotype 04    .......... ........T. .......... .......... .......... .......... .......... .......... ..........
Haplotype 05    .......... ........T. ...C...... .......... .......... .......... .T........ .......... ..........
Haplotype 06    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07    .......... ........T. .......... .......... .......... .......... .......... .......... ..........
Haplotype 08    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11    .......... ........T. ...C...... .......... .......... .......... .......... .......... ..........
Haplotype 12    .......... ........T. .......... .......... .......... .......... .......... .......... ..........
Haplotype 13    .......... ........T. ...C...... .......... .......... .......... .......... .......... ..........
Haplotype 14    .......... ........T. ...C...... .......... .......... .......... .......... .......... ..........
Haplotype 15    .......... ........T. ...C...... .......... .......... .......... .......... .......... ..........
Haplotype 16    .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                    370        380        390        400        410        420        430        440        450
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01 GGCCGCTTAA TCACCATGCC GCGTGAAATC ATCAACCCGC CCACAACGTG TCCCTCTTCT CGCTCCGGGC CCATATGGAC TGTGGGGTAG
Haplotype 02 .......... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 03 .......... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 04 .......... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 05 ......C... .......... .......... .C........ .......... .......... .......... ......A... ..........
Haplotype 06 .......... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 07 .......... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 08 .......... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 09 .......... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 10 .......... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 11 ......C... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 12 .......... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 13 ......C... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 14 ......C... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 15 ......C... .......... .......... .......... .......... .......... .......... ......A... ..........
Haplotype 16 .......... .......... .......... .......... .......... .......... .......... ......A... ..........

                    460        470        480        490        500        510        520        530        540
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01 TTATAGGAGA ACTATACCTG GCATCTGGTT CTTACTTCAT GTTCATTCGT CTATAGTCGC TCACTCGTTC CTCTTAAATA AGACATCTCG
Haplotype 02 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05 .......... .......... .......... .........C .......... .......... .......... .......... ..........
Haplotype 06 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 13 .......... .......... .......... .........C .......... .......... .......... .......... ..........
Haplotype 14 .......... .......... .......... .........C .......... .......... .......... .......... ..........
Haplotype 15 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 16 .......... .......... .......... .......... .......... .......... .......... .......... ..........
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APPENDIX D

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                     550       560       570       580       590       600       610       620       630
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01 ATGGATTAGT TACTAATCAG CCCATGCCGA TGCATAACTG TGGTGTCATG CCCTTGGTAT TTTTTAATTT TAGGGTGCGG GGGTTCCACT
Haplotype 02 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09 .......... .......... .......... ..G....... .......... .......G.. .......... .......... ..........
Haplotype 10 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 13 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 14 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 15 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 16 .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     640       650       660       670       680       690       700       710       720
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01 AGGCGGAGCC TCAGAGCGGA GGACATTGAA TGTGGTCAAG ACATATATTT AACTATTATT GGTCGTACAT ATACTACTTT CAGGTGTTAT
Haplotype 02 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09 .......... ..G....... .......... .....G.... .......... .......... .......... .......... ..........
Haplotype 10 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 13 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 14 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 15 .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 16 .......... .......... .......... .....G.... .......... ..G....... .......... .......... ..........
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                        730       740       750       760       770       780       790       800       810
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01    TCAGTCAATG GTTACGGGAC ATATAAAATT TTACTACAAC ATTTCAAGTC AACAATTTTT CCATCATACA TATAAATTTA TTTTACGTAG
Haplotype 02    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05    .......... .......... .......... .......... .......... .......... ....T..... .......... ..........
Haplotype 06    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08    .......... .....A.... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12    .......... .....A.... .......... .......... .......... .......... .......... .......... ..........
Haplotype 13    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 14    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 15    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 16    .......... .......... .......... .......... .......... .......... .......... .......... ..........

                        820       830       840       850       860       870       880       890       900
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01    CTTAGTATTC AAACCCCCCC TTACCCCCCC AATCTTACAT GTACTAAACA TCTTGTCAAA CCCCAAAAGC AAGAATACAT ACACTGTAAG
Haplotype 02    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05    ....A..... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12    .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 13    ....A..... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 14    ....A..... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 15    .......... .......... .......... .........C .......... .......... .......... .......... ..........
Haplotype 16    ....A..... .......... .......... .........C .......... .......... .......... .......... ..........
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                      910       920       930       940       950       960       970       980       990
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  TGCTACAAAA AACATAAAGT ATTACGTAAG CATTCACGTA AGCATTTGGC TCAAACCTTG TTACACAGTT TTCTGCCTGC TCTAAAAACA
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 13  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 14  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 15  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 16  .......... .......... .......... .......... .......... .......... .......... .......... ..........

                     1000      1010      1020      1030      1040      1050      1060      1070      1080
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Haplotype 01  CTTTATTGTT TGGACATATA TGATCTGGAA ATCTTATAGT AATTATAGTA TTAGAATATT TTATTAATGT AACATAGAGA ATATAATTTT
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 05  .......... .........G .......... .......... .......... .......... .......... .......... ..........
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 07  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 10  .......... .......... ......A... ..G....... .......... .......... .......... .......... ..........
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 13  .......... .........G .......... .......... .......... .......... .......... .......... ..........
Haplotype 14  .......... .........G .......... .......... .......... .......... .......... .......... ..........
Haplotype 15  .......... .......... .......... .......... .......... .......... .......... .......... ..........
Haplotype 16  .......... .......... .......... .......... .......... .......... .......... .......... ..........
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                     1090       1100       1110       1120       1130       1140       1150       1160
              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
Haplotype 01  TAAACCTACG CAAAATATTT ACGCATTTAT TTATTCTTAT AGCGGCATAA TGCAAACTAT CTGCTCCCTA CATGCAAATT AAA
Haplotype 02  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 03  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 04  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 05  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 06  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 07  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 08  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 09  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 10  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 11  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 12  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 13  .......... .......... ...T...... .......... .......... .......... .......... .......... ...
Haplotype 14  .......... .......... ...T...... .......... .......... .......... .......... .......... ...
Haplotype 15  .......... .......... .......... .......... .......... .......... .......... .......... ...
Haplotype 16  .......... .......... .......... .......... .......... .......... .......... .......... ...
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