DNA Barcoding as a Tool for the Identification of Illegally Traded Wildlife Products

Alphonse R. Nougoue

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements
For the Degree of Master of Science (Biology) at
Concordia University
Montreal, Quebec, Canada

May 2012

© Alphonse Nougoue, 2012

CONCORDIA UNIVERSITY

School of Graduate Studies

This is to certify that the thesis prepared

By: Alphonse R. Nougoue

Entitled: DNA Barcoding as a Tool for the Identification of Illegally Traded Wildlife Products

and submitted in partial fulfillment of the requirements for the degree of

Master of Science (Biology)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

_____Chair
Dr. D. Fraser

_____Examiner
Dr. J. Grant

_____Examiner
Dr. S. Dayanandan

_____External examiner
Dr. D. McLaughlin

_____Supervisor
Dr. D. Hickey

Approved by Dr Selvadurai Dayanandan
Chair of Department or Graduate Program Director

2012 Dr Brian Lewis
Dean of Faculty

Abstract

The rapid identification of species using DNA barcoding technology could facilitate the monitoring of the trade in wildlife products. A short fragment of mitochondrial DNA sequence has been established as a standard DNA barcode for animal species identification. We tested the utility of these DNA barcodes using taxonomically verified samples obtained from two Canadian Zoos. Then we tested the feasibility of getting DNA barcode sequences from various "field samples" that would mimic the conditions of confiscated wildlife products. We also asked whether designing more specific primers for DNA amplification might increase the success rate of obtaining DNA barcode sequences from commonly hunted mammalian species. Five of the major animal orders involved in the wildlife trade were included in our study. Our results show that we were able to obtain high quality DNA barcode sequences from almost all samples. We were able to amplify DNA from the blood samples from the Zoos and to get high quality DNA from fresh, smoked and processed meat samples purchased from grocery stores and restaurants. Only a few samples, specifically old hair from dried animal skins, failed to yield amplifiable DNA. We conclude that: (i) DNA barcodes provide a rapid and reliable method for species identification when applied to wildlife trade monitoring, and (ii) this technique is applicable to samples collected under field conditions, such as those that might be obtained when monitoring the bushmeat trade.

Keywords: Mitochondrial cytochrome c oxidase I (mtCOI), Wildlife monitoring, intraspecific divergence, DNA barcoding

Acknowledgements

I would first like to acknowledge and thank Dr. Donal Hickey for his support and guidance throughout my MSc degree. I thank committee members, Dr. Selvadurai Dayanandan and Dr. James Grant for their advice and comments on preliminary draft of thesis. I also acknowledge Dr. Mehrdad Hajibabaei for accepting me as a visiting student at the Science Complex of the University of Guelph last summer. I particularly thank Dr. Hajibabaei and his lab for their support throughout this work and the Canadian Centre for DNA Barcoding (CCDB) for providing the mini barcode primers for hair from old skin samples. This project was undertaken in collaboration with the Granby and Toronto Zoos from where blood and hair specimens were obtained. I thank both Patrick Pare of the Granby Zoo and Gabriela Mastromonaco of the Toronto Zoo for their help and support. I thank Dr. Paul Donfack for providing a list of hunting regulations from the Ministry of Forest and Wildlife (MINEF) of Cameroon. Special thanks go to the McLaughlin and Hickey labs for their help and other support. I lastly thank my family for their encouragement throughout these investigations.

TABLE OF CONTENTS

List of figures

List of tables

List of abbreviations

BOLD	=	Barcode of Life Data system
BLAST	=	Basic Local Alignment Search Tool
CCDB	=	Canadian Centre for DNA Barcoding
CITES	=	Convention on International Trade in Endangered Species
COI	=	Cytochrome Oxidase I
EN	=	Endangered
IUCN	=	International Union for the Conservation of Nature
MINEF	=	Ministry Of Wildlife and Forestry
MtDNA	=	Mitochondrial DNA
NCBI	=	National Centre for Biotechnology Information
NT	=	Near Threatened
NJ	=	Neighbor Joining
NC	=	Negative Control
NuDNA	=	Nuclear DNA
OD	=	Optical Density
PCR	=	Polymerase Chain Reaction
PubMed	=	Public Library of Medicine
SD	=	Standard Deviation

Chapter I: Introduction

I.1- The illegal trade in animal products

Historically, the hunting of wildlife species in humid tropical regions has mainly been for subsistence consumption and local trade. Millions of people in Asia, Africa and South America have relied on harvested wildlife products as a source of food and income (Bennett et al. 2002; Milner-Gulland and Bennett 2003). The wild meat trade can be viewed in a "highly positive sense as one of the great success stories of autonomous food production in the developing world, and a testimony to the resilience and self sufficiency of its populations" (Brown 2003). During recent years, however, the overhunting of bushmeat has become a global crisis. Both local communities and foreign commercial interests are focusing on bushmeat and other products not only for food but also the development of medicinal products, both traditional and modern (Alves and Rosa 2005). Consequently, the massive overhunting of tropical wildlife populations is a growing threat to the survival of many animal species. The result is that many large and medium-sized animals, including those on the International Union for the Conservation of the Nature (IUCN) list of endangered species, are experiencing local and global extinction because of the illegal trade in their products (Kohn and Eves 2006). One of the main constraints in controlling this illegal trade is the difficulty in identifying many suspect animal products at the species level. This difficulty is even greater in cases of imported products of unknown origin. The lack of information about the species identity and origin of many morphologically similar products (such as meat) makes it extremely difficult to control the bushmeat trade. This threatens not only the survival of several wildlife species, but it also threatens the future of many local communities whose livelihood depends on the wildlife resources. Further, the

increasing loss of animal species could eventually lead to overall ecosystem instability. The threat is especially serious for a number of species, including chimpanzees, gorillas, forest elephants, small antelopes (i.e. duikers) and numerous monkeys (Hance 2011). For instance, in 2010 the IUCN suggested the possibility of local extinction of black rhinoceros in West Africa following its extirpation in Cameroon (IUCN 2010). The remaining subspecies of black rhinoceros that still survive in East and South East Africa are also at risk. Rhinoceros have been hunted for centuries for trophies and for meat. Their skin has been used for shields and good luck charms and their horns have been used in traditional medicines (Emslie and Brooks 1999).

Rural communities in the tropical rainforest rely on hunting for food and income, and for most people, the consumption of domestic livestock products is not an affordable option. Indeed, due to high production costs farming of livestock is impractical and, consequently, domestic meat is expensive. In addition, both taste and food habits appear to be two major reasons that can explain preference for bushmeat even when domestic meat is available (Mbete et al. 2011). For instance, urban dwellers who grew up in rural areas often prefer bushmeat to domestic meat regardless the cost. This has led to the export of local meat from rural areas to urban centres. The bushmeat issue has therefore acquired an international dimension since most of the human migration moves from the South to the North, thus generating an illegal flow of meat products in the same direction. There are a number of reasons that emigrants choose to eat bushmeat but the most important seems to be the taste according to the majority of survey respondents (Hance 2011); the same survey also found that urban dwellers born in rural areas preferred bushmeat for its "rooted cultural value" (Hance 2011).

Another major contributing factor to the large-scale destruction of wildlife is the improvement of hunting technology. This acts in combination with the increasingly developed road infrastructure which facilitates illegal access to wildlife in forests that were once remote. Many of these roads are established and maintained by logging concessions and these have provided hunters greater access to relatively unexploited populations of forest wildlife and have lowered the cost of transporting bushmeat to market (Hance 2011). In addition, local human population growth, habitat loss, and urbanization have also led to the increased illegal killing and trade of wild animals in these regions. As the human population continues to grow and poverty increases, increasing numbers of people are becoming dependent on bushmeat for food and also for the income opportunities from the trade in wildlife products (Bowen-Jones and Pendry 1999; Milner-Gulland et al. 2003). Although these reports clearly reveal an alarming picture, one of the immediate problems in dealing with the bushmeat trade is the inability of most state agencies to efficiently monitor the illegal killing of wild animals. The overexploitation of wildlife products is also facilitated by the higher profits that are made by the commercial wildlife traffickers, often working with international commercial networks, who supply local and international markets with various meats and other animal products for the traditional medicine and other kinds of practices. Large animals with low reproductive rate are most susceptible to overexploitation compared with smaller species that apparently can tolerate relatively intensive hunting (Chapman et al. 2006).

The major animal activities and products associated with the commercial trade are: commercial trophy hunting, animal skins, teeth, fresh, smoked and processed meat, bone, blood, and scales. Previous research in the Congo basin revealed that the volume of extracted bushmeat has increased considerably and many species are in sharp decline due

to overexploitation (Albrechtsen et al. 2007; Bennett et al. 2002; Milner-Gulland and Bennett 2003; Redford 1992). Sometimes there is conflict between farmers and animals that damage farmers' crops causing farmers to kill problem animals. For example, rodents damage crops more than other wildlife species in subsistence agriculture on the northern periphery of Dja Reserve Cameroon (Arlet and Molleman 2007). Humans also kill carnivores that compete for the same game, such as herbivores, rodents and primates. Mbete et al. (2011) reported that the top three orders of mammals for meat were artiodactyls (48%), rodents (28.3%), and primates (13.0%). There is often, particularly in West and Central Africa, a strong interplay between the trade in bushmeat and its control, and this may take the form of sanctions against illegal hunting (Bennett 1995).

Despite efforts made by the Convention on International Trade in Endangered Species (CITES) to deal with this crisis, the illegal trade in animal products continues to expand. Recent reports by the China Xinhua News revealed that three people were imprisoned for up to ten years in jail, for illegally trading ivory and rhinoceros products. In 2009, 377 kg of ivory and 137.07 kg of rhinoceros horn products were found in one of the convicted trader's apartment. Unfortunately, many products seized from illegal traders at market stalls or confiscated as illegal imports are not identifiable and their origin is often unknown. Further, it is difficult to distinguish between smoked domestic and wild meat products and to determine which came from an endangered species (Eaton et al. 2009). DNA tests by researchers from the University of Washington in the United States of America in 2007 revealed that many seizures made in Singapore in 2002 came from Zambian elephant populations (Wasser et al. 2008). Clearly, accurate species identification is still a major impediment in the regulation of commerce involving endangered species.

I.2- DNA barcoding technology

A new molecular technology known as DNA barcoding was developed in 2003 by Dr. Paul Hebert at the University of Guelph (Hebert et al. 2003a, 2003b). It is now used as a standard tool for the rapid identification of animals and plants at the species level. The DNA barcode for animals is a 658 base pair fragment of the mitochondrial COI gene. This sequence mutates quickly enough to distinguish closely related species but slowly enough that individuals within a species have similar barcodes (Brownlee 2004). Once a universally available database is established for these sequences, it should help to control illegal use of meat and product from endangered species (Ratnasingham and Hebert 2007).

I.3- Research strategy

The overall objective of this study was to test the hypothesis that bushmeat in its various forms can be identified to the species level using DNA barcoding and particularly to assess the applicability of this technology in biodiversity management in the tropics. A number of species are listed as endangered by the Cameroon Ministry of Wildlife and Forestry (MINEF 1998). We were able to obtain blood samples from several of these from species currently held in the Toronto and Granby Zoos as well as samples from other species held at these facilities. We also tested samples of various other animal products that had been subjected to various treatments such as fresh, boiled, smoked, and processed samples, often from unknown origin. These animal products were acquired from various grocery stores and restaurants.

We assessed the level of efficiency of different commercial DNA extraction kits to extract DNA from dried blood samples obtained from various specimens. These

consisted of samples preserved on the FTA cards (Whatman, New Jersey), which can be stored at room temperature for several weeks and months without the need of refrigeration. Then, the efficacy of the primer cocktail for vertebrates proposed by Ivanova et al. (2006) was tested for amplification of DNA the feasibility of amplifying DNA sequences from various samples available. These consisted of various fresh, cooked, smoked partly degraded and processed tissue samples for which species origin was sometimes unknown, were acquired from various grocery stores and restaurants for additional analysis and classification. Because previous studies have described the development of specific primers for fish that worked, we developed specific primer pairs per order of mammal in order to supplement the primer cocktails for vertebrates available in the literature. We designed these primers using the mammal tree of life to find sequences of different species alignments from BLAST searches on specific mammalian orders in Genbank using Geneious software (Drummond et al. 2009).

Also, the universal mini-barcode primer pair (Meusnier et al. 2008) was tested to determine if the DNA extracted from hair samples of old skin specimens could be amplified since the regular DNA barcode fragment could not be amplified from this highly degraded material. A particularly important application of mini-barcodes lies in its ability to obtain sequence information from old degraded specimens (Meusnier et al. 2008).

Chapter II: Materials and Methods

II.1- Sources of Biological Samples

Several species of animals from 5 major mammalian groups were studied: Artiodactyla, Carnivora, Perissodactyla, Primates, and Proboscidae. Blood samples were collected from 36 different individuals from Toronto Zoo, Ontario and 24 different individuals from Granby Zoo, Quebec. In addition, hair and blood samples from two human specimens as well as hair from four old skin specimens from Granby zoo were also included.

Most of the specimens obtained from the Toronto Zoo were from species originally from the African Congo basin while those from the Granby Zoo were from South and Central America, Asia and Australia. Reference data for all specimens (e.g. specimen number, geographical origin, etc.) were also recorded. Different meat samples were purchased at various local grocery stores and restaurants. The species origin of some of these purchased samples was unknown.

Two groups of specimens were designated throughout this study. The first group ("known specimens") includes all taxonomically verified samples from zoos. These consisted of blood samples acquired from zoo animals collected on the Whatman FTA cards. Other samples included animal hairs. The second group consisted of meat purchased at local grocery stores (Metro, Esposito, and Provigo), restaurants (smoked and spicy pork labeled as "Jambon cru de champagne", "Procuito parma" and "Keiser fleisch") and kangaroo meat from , "La Maison du Gibier" (www.lamaisondugibier.com), one of the largest game meat processor in Canada .. Also included in this group were some of the

local meat samples prepared in my own kitchen (boiled, cooked and grilled). . The number of specimens examined is summarized by order in **Figure 2.1**.

II.2- Collection and storage methods

Blood samples were preserved on Whatman FTA cards in order to confirm the feasibility of the preservation of biological samples at room temperature. Other known specimens were collected on the labeled Whatman filter cards with the specimen's name and reference number. FTA cards are eminently suitable for collection and purification of nucleic acids from biological samples from a wide range of species (Smith and Burgoyne 2004). This technology makes the collection and storage of such samples much easier. Other samples e.g. purchased meats, and hair samples Samples were collected and preserved individually in labeled plastic bags and stored in a freezer. Many other samples obtained from fresh, boiled, smoked grilled and processed specimens were collected and put in the labeled plastic bags but not all species origin's information was provided. Old and fresh hair samples were also put in the plastic bags with their reference information.

Freezing is the standard storage method for DNA samples; such samples need to be kept at (-20^{o}C) to avoid any degradation risk but for archive samples or longer storage period over several years, it is better to store at (-80^{o}C). The FTA cards contain chemicals that protect biological samples against degradation and contamination for over several months at room temperature. This allows the preservation of the DNA at any time, in a space-efficient way and at a low cost since samples can be transported by mail or by personal baggage (Smith and Burgoyne 2004). This technique does not completely replace the refrigeration; rather the idea is to allow collection in the field. Overall, samples of 70

specimens were preserved on the FTA cards and stored at room temperature. The cards were first air-dried and then put in bags with silica-gel desiccant.

II.3- DNA extraction method

DNA was obtained using two extraction kits, Qiagen and Nucleospin, following the manufacturers' respective protocols. The Qiagen kit was mainly used for DNA extraction from all fresh tissue samples (obtained from the grocery stores while the Nucleospin extraction kit (purchased at Macherey-Nagel Inc), was mainly used for DNA extraction from dried blood samples preserved on the Whatman cards after several weeks. To increase the chance of a successful DNA extraction, all the samples were decontaminated using Ethanol 99%, DNA Eliminase or distilled water before any extraction process. Eliminase especially was used to remove unwanted DNase; this product was purchased at Decon labs. Inc.

Hair samples were treated in liquid nitrogen and thawed in the 56°C water bath several times, afterward about 100 shaft hairs and follicles were put in the labeled collection tubes prior to DNA extraction. DNA elution was performed in Nucleospin buffer BE containing 5mM Tris/Hcl, PH 8.5. DNA concentration was assessed using NanoDrop ND - 1000 spectrophotometer (Thermo Fisher Scientific)

II.4- COI amplification

II.4.1- pre-amplification

I.4.1.1-Primer design

Primers are short, single-stranded DNA molecules with up to 26 bases that bind the targeted DNA at the starting point and are copied by the enzyme for subsequent syntheses. The specific primer cocktails (forward and reverse) for vertebrates proposed by Ivanova et al. (2006) were tested for the amplification of the barcode COI sequences of most of the samples available (blood and various meats). We also tested three different sets of Ivanovas' primers during our preliminary work on field samples to find the most reliable ones for DNA amplifications (Figure 2.2). The primer pair with inosine (Vf1i and Vr1i) failed to amplify DNA from most of the field samples while the primer pair Vf1d and Vr1d always provided successful COI amplification. New primers for different taxa were therefore designed based on the regular Ivanova et al. (2006) primers (Vf1d and Vr1d).

Previous studies have investigated the development of specific primers for fish to test the variation between species. Approximately 600 base pair (bp) of the 5' region of the COI gene were amplified using the primer pair FishF2 and FishR2 (Ward et al. 2005). We developed new primers for specific groups for accurate species identification to support the primers from the literature. We found the primer pair (Vfd1/Vrd1) more reliable for designing new primers based on the preliminary tests. We therefore aligned these primer pairs with the COI gene region of five different species from each category of mammals (Artiodactyls, Primates, Carnivore, etc) in order to initiate their own primers. The COI sequences of various species were obtained from Genbank using NCBI Blast tool. The Geneious software computer package was used to perform the alignment of DNA

sequences in this region of similarity between species and the regular primers from the literature (Meintjes et al. 2011). New primers generated for specific vertebrate groups are shown in **Figure 2.3**.

II.4.1.2-Primers for amplifying DNA mini barcodes

A mini primer is a set of 26 base pair sequences designed to target a short region of DNA from highly degraded biological and forensic samples. These have been successfully used to amplify a 177 bp fragment of the mitochondrial control region in cattle (Vuissoz et al. 2007). The mini barcode primers amplify a 100-200 base pair region when a full barcode cannot be obtained from a particular specimen.

We used mini primers: 5'-TCCACTAATCACAARGATATTGGTAC-:

5'-GAAAATCATAATGAAGGCATGAGC-3' (Meusnier et al. 2008) in attempt to amplify old hair samples.

II.4.1.3-PCR Master Mix

The PCR master mix preparation was done in accordance with the Bioshop protocol (Bioshop Canada. Inc., TAQ001.1). Each PCR reaction consisted of: 17.5µl sterilized distilled H_20, 2.5µl 10X (-MgCl2) PCR buffer, 1.5µl $MgCl_2$ (25mM); 0.125µl dNTP (10mM; Fermentas #R0191), 0.25µl of the forward and reverse PCR primers and 0.125µl Taq DNA polymerase (Bioshop Canada.Inc., TAQ001.1) and 3µl of DNA template.

The 10XPCR reaction buffer supplied with the Taq DNA polymerase consisted of 200mM Tris-HCl (pH 8.4), 200mM KCl, and Tween 20 and enzyme stabilizers. Indeed, the full length primer pair from the literature (Vf1d/Vr1d) was used to amplify the targeted 658

base pair region of the mtCOI gene of tissue samples available including those from the FTA cards. In addition, the specific primers designed were used for assessing DNA sequence from meat samples at different level of treatments. Another protocol from CCDB designed especially for old tissues samples was also performed. We used mini barcode primer pairs for amplification between 100-300 base pair as the chemicals used for their treatment might affect the amplification of the full region. Contaminations from other sources may lead to difficulties in extraction DNA from old tissue samples. Thus, for old hair samples, 10.5µl per reaction was used (10.3µl PCR mix; 0.125µl mini forward primer; 0.125µl mini reverse primer) plus 2µl of DNA stock. In order to minimize contaminants, DNA stock was diluted at different concentrations (2.0µl stock; 1ul stock plus 9.0µl water; 1ul stock plus 99µl water; 1µl stock plus 999µl water).

II.4-2- PCR amplification process

A standard PCR pre-mix was used for the PCR reaction. Amplification protocol was as follows: 94^{o}C for 1 minute, followed by 5 cycles of 94^{o}C - 30 seconds, 50^{o}C- 40 seconds, and 72^{o}C- 1 minute, followed by 94^{o}C- 30seconds, 55^{o}C- 40 seconds, 72^{o}C- 1 minute, and finally a final extension 72^{o}C- 5 minutes. We included one negative control reaction (without DNA template) in our PCR 96- plates.

II.4-3- Amplification product verification

The amplification products were visualized on a 1% agarose gel (Invitrogen, Burlington, Ontario) to determine which amplifications were successful. A molecularladder

(Fermentas: GenRuler^{TM} 100bp plus DNA Ladder # SM0321) was used to determine the size of the product.

II.5- DNA sequencing

Most of the samples were sequenced at the University of Guelph Genomic Centre while the remaining samples were sequenced at Genome Quebec Innovation Centre at McGill University.

II.6- Data analysis

The forward and reverse sequences of each sample were edited and a contiguous sequence produced using Geneious version 4.75. Sequences and original trace files are available on BOLD (http:// www Barcodinglife.org) and Genbank. The resulting sequences were compared with the sequences and original trace files. Sequences not available in the BOLD database or showing more than 1% of divergence with the closest species were checked in GenBank (Avise 2000). The BLAST program finds regions of similarity between species. The assessment of the intraspecific and interspecific variations was performed using BLAST searches in GenBank. Results from the databases were used to assess the difference in nucleotide divergence within and between closely related species. The ClustalW program, available in MEGA version 4 (Tamura et al. 2007) was used to build multiple sequence alignments and to build the phylogenetic tree. About 70 sequences from Zoo samples were analyzed using MEGA version 4. The Neighbor Joining (NJ) method was used to build the phylogenetic tree for inferring the evolutionary

relationship between species (Saitou and Nei 1987). The original data sets were bootstrapped to determine the strength of the relationship of one taxon or group of taxa compared to another.

Chapter III: Results

For presentation purposes, we divided the samples into two groups. The first group comprises the samples obtained from zoos (taxonomically verified samples), and the second group is for samples obtained from restaurants and grocery stores.

III.1- Taxonomically verified samples

We obtained samples from 60 specimens out of 61 (36 blood samples from the Toronto Zoo, 23 samples from Granby Zoo and 1 from human blood). All of the dried blood samples were preserved on the FTA cards. These specimens represent 29 of morphologically defined species spanning 8 different Orders (Carnivora, Artiodactyla, Primates, Proboscides, Perissodactyla, Xenarthra, Diprotodontia, Struthioniforme). Fifteen species out of 29 were from Toronto Zoo while 14 species were from Granby Zoo. Approximately one quarter of the taxa from both Zoos were represented by a single individual; the other three quarters of the species were represented by two or more individuals. . Amplification of guinea pig blood from the Granby Zoo failed to amplify. Other category of known samples included 4 old animal skins from the Granby Zoo (Gorilla, leopard, lemur, and giant panda old hairs) and 1 human hair.

Results from agarose gel visualization confirmed the reliability of both Qiagen and Nucleospin extraction kits for DNA extraction from dried blood samples stored at room temperature for several weeks **(Figure 3.1).** This was confirmed using DNA quantification **(see Figure 3.2)**. All samples obtained from the Toronto Zoo were successfully sequenced **(Figure 3.3).** Among the samples obtained from the Granby Zoo, all DNA extractions and

subsequent PCR amplifications were also successful, except for the sample collected from the Guinea pig **(Figure 3.4**; well 23). Two repeated procedures with Nanodrop machine confirmed the presence of DNA in this sample suggesting that the DNA failed to amplify. Results from the intraspecific comparison of our cheetah barcode sequences with the Genbank database showed perfect match for *Acinonyx jubatus* (**Figure 3**.**5**). Comparing two closely related species to find the interspecific variation (*Lynx canadensis vs. Lynx rufus*) we found 91% of DNA sequence of identity (**Figure 3.6)**. This confirmed the ability to discriminate between closely related species using the barcode COI.

Results from the analysis with BOLD database system comparing the Emu DNA sequence showed perfect match for the DNA barcode sequence from *Dromaius novaehollandiae* (an Australian bird). We found a perfect match for almost all of our taxonomically verified dried blood samples analyzed with Geneious and blasted in GenBank database.

Blast search for Africanelephant2 barcode sequence in the GenBank database (**Figure 3.10)** matched perfectly the forest elephant *Loxodonta cyclotis* with 99.56% similarities, the nearest other extant species match being *Loxodonta africana* with 96.70% similarities. **Table 3.1** displays the percent of similarities between the africanelephant2 barcode sequence and various Proboscidea sequences in the Genbank database. Indeed, Africanelephant2 sequence was also compared with DNA sequences from Asian elephant and the extinct *Mammuthus primigenuis* (Table 3.1, **Figure 3.11).** Using the same tool, we then compared our sequence with the Asian elephant *Elephas maximus* and found 95% of identities **(Figure 3.12)**. The difference in geographic location can explain the genetic variation between elephant at the species level. So the more the difference, the higher is the genetic variation.

Table 3.1: Relationship between elephant species based on the barcode COI sequences. Above the diagonal line, the percent sequence identity is shown; the values below the diagonal are the percent sequence difference. These values are obtained from the search of the level of interspecific relationship between species in Genbank.

	Loxodonta cyclotis	*Loxodonta africana*	*Mammuthus primigenius*	*Elephas maximus*
Loxodonta cyclotis		96.70	96.48	94.72
Loxodonta africana	3.30		94.86	94.89
Mammuthus primegenius	3.52	5.14		95.27
Elephas maximus	5.58	5.11	4.73	

But, old hairs failed to yield full barcode sequences although the mini barcode region was amplified successfully in all of them. None of the old samples matched their expected sequences possibly due to contamination. Also using ClustalW program version MEGA.4 to perform the multiple alignments, we were able to find the phylogenetic relationship between our DNA barcode sequences. The Neighbor Joining method was used to group similar sequences together and the default parameter values were used for the determination of the genetic divergence within and between species **(Figure 3.7).** We bootstrapped the phylogenetic tree to make a tree that retained only the statistically significant nodes. Nodes with bootstrap support of less than 70% were collapsed. The resulting tree showed that in general only the nodes at a shallow phylogenetic depth are well supported (**Figure 3.8).**

A graphical view of these sequence relationships is shown in a radial phylogenetic tree (**Figure 3.13**).

III. 2- "Field" samples

Sixtheen specimens from various treatments were tested.. Several samples were obtained from 8 specimens (2 pork meat and liver; 2 beef meat and liver; 1 chicken; 1 fish; 2 bison meat and skin) purchased from the grocery stores and restaurants. Other samples were obtained from 8 unknown specimens purchased from the restaurants. These meat samples from the grocery stores and restaurant were of different types (cooked, fresh, smoked, processed, and dried). However, the treated samples, with the exception of a single partly degraded sample of unknown"meat juice A", yielded full barcode sequences. The DNA sequences of these products matched perfectly with the respective species' sequence in the

GenBank database. Two of the unknown specimens (dried and juice D) matched kangaroo in GenBank (99.3% for *Macropus robustus*).

III. 2.1- DNA quantification

In order to assess the amount of DNA concentration from various sample types, we used Nanodrop spectrophotometer. The amount of DNA collected from fresh tissue samples was similar to those from cooked, smoked, and processed meat samples. Results from the preliminary test on fresh, cooked, smoked and processed samples ranged between 3- 32ng/µl for an equal volume extracted (the Genome Quebec Innovation Centre document suggested a range between 5-20ng/µl). Using Nanodrop we found grilled sausage (mixed meat) had the highest DNA concentration (31.48ng/µl) and the OD values (OD260/OD280 = 1.64; OD260/230 = 0.70) obtained. The amount of DNA needed for a full length barcodes was obtained but the 260/280 ratio (optical density) was close to 1.7 (OD260/OD280 = 1.64) and the 260/230 ratio was less than 1.7 (OD260/OD230 = 0.70). As the value 2 was 0.70 lower than 1.7, this indicated potential contamination of DNA stock by salt while value 1 was close to 1.7 meant very little protein contaminants. The *Gorilla gorilla* hair sample showed 0.96ng/µl for a total volume of 50µl though the mini barcode primers amplified successfully the 130 bp mtCOI gene region of the DNA stock. Moreover, the amount of DNA extracted from old hair samples was very low (the document mentioned above suggested a range of 1-3ng/µl). The amount of DNA needed for a short length barcodes was obtained but the 260/280 ratio was less than 1.7 (OD260/OD280 = 1.29) and the 260/230 ratio was less than 1.7 (OD260/OD230 = 0.46).

III.2.2- Primer selection and analyses

III.2.2.1-Amplification results with new primers

The amplifications with the specific primers for each taxon was successful. A test with the Artiodactyls primers is shown in **Figure 3.14**. The other primers designed here were equally successful. Various analyses with Geneious and BLAST showed perfect match for the corresponding barcode sequence in Genbank.

III.2.2.2-Amplification results with primer cocktails

Our preliminary test on fresh and boiled pork, beef, fish and chicken with the general primers (VfId/VrId) yielded the strongest bands. Treated samples (i.e. fresh, frozen, smoked, grilled, dried, and processed samples) were successfully amplified with full barcode primer cocktails provided by the CCDB and sequenced and their species origins determined. The only samples that failed were those of old hairs from specimens acquired at the Granby Zoo **(Figure 3.15).** However the test on old hair COI amplifications with mini barcode primers was successful only for the regular DNA stocks while all 1/100 diluted DNA stocks failed (**Figure 3.16).**

III.2.2.3- COI amplification of unknown samples

Unknown samples of meat juices preserved on the FTA card as well as dried meats stored at room temperature, all from restaurants, were successfully amplified with full barcode primers and their species origins determined (**Figure 3.17**). The fresh samples matched *Gallus gallus* in Genbank as the nucleotide sequences of the reverse chromatogram match perfectly those of the forward chromatogram **(Figure 3.19)**; while two fresh and boiled chicken DNA sequences from the same specimen showed different

percent identities in the database after several repeated tests (**Figure 3.18)**. The accuracy of an alignment is not only based on the green band from the consensus identity but also on the similarity of complementary nucleotides.

A troubleshoot during the pairwise alignment process in Geneious can affect the species level match in Genbank. For example, on the chromatogram in **Figure 3.19**, we see that the sequence from the forward primer showed good sequence quality at the beginning of the sequence and poor sequence quality at the end, while the chromatogram for the reverse primer showed poor quality sequences at the beginning and well defined sequences at the end as most sequences behave this way. By using the good quality sequence from each, we can correct the poor quality sequence from the complementary strand. So, looking at both chromatograms in **Figure 3.18**, we can choose the sequence (A) instead of the sequence (T) and at the 27^{th} position we expected the sequence (T) instead of (C).

In addition to sequencing errors, there were also some sequence differences between different specimens within the same species. This very low level of sequence variation within species can be explained by the level of polymorphism occurring happening when comparing two specimens in order to assess the percentage of divergence between both in GenBank (F**igure 3.20).** Multiple sequence alignments were used to generate Neighbor Joining phylogenetic trees. The results from the tree showed a clear discrimination between the samples **(Figure 3.21).** All of meat samples, including those without any identifying information, were successfully identified to species level.

We did not include old hair samples though regular DNA stocks amplification were successful. Although BLAST analysis indicated a match for *Bison bison* in GenBank, subsequent repeated tests with fresh and grilled sausage the same day in the same lab

matched for *Gallus gallus.* All bootstrap values less than 70% were once again neglected for the effectiveness of specimens' distribution **(Figure 3.22).**

III.2.3- Sequence variations

Overall the nucleotide variation within species was generally very low (< 1%) using mtCOI as DNA barcode; an example is shown in **Figure 3.5**. The nucleotide variation between closely related species was more than 2% (**Figure 3.6**).

Chapter IV: Discussion

IV.1 – The utility of DNA barcoding

This study reinforces the utility of DNA barcoding as a bridge between species identification and wildlife conservation. The application of this tool has proven useful for the discrimination of vertebrates (i.e. bird, fish) and various invertebrates (i.e. crustaceas) at the species level. The development of new primers by taxon and multiple analysis using bioinformatic tools are increasing the success of the barcoding technology as many researchers are now being interested and in turn many species are barcoded. However, a specimen's barcode is only considered when valid references are available. The effectiveness of DNA barcoding technology has been validated for various animal groups and most investigated species (> 94%) possess distinct barcode arrays, with low intraspecific variation and high divergences from closely allied taxa (Ward et al. 2005; Hadjibabaei et al. 2007).

Increasing the database may lead to an efficient control of the wildlife trade. We obtained an accurate pair wise alignment of African elephant 2 (**Figure 3.9),** but the results from BLAST search of this elephant barcode sequence matched only 97% *Loxodonta africana* in Genbank, representing 3% of dissimilarity. A comparison with Asian elephants and the extinct Mammoth confirmed this animal to be originally from Africa as shown in the tree (**Figure 3.14)**. A recent extensive study of elephant providing lots of mitochondrial data on forest elephant confirmed the percentage difference between elephant species (Brandt et al. 2012). Indeed, using fossil calibration, they found the divergence between the two African elephant mitochondrial genomes (forest clade and savannah clade) to be

estimated as 5.5Ma (Brandt et al. 2012). The level of divergence between the (African) ancestor of the mammoth and Asian elephant lineages was estimated to be 6.0 Ma, indicating that four elephantid lineages had differentiated in Africa by the Miocene-Pliocene transition, concurrent with drier climates (Brandt et al. 2012). Our results confirmed the existence of four different groups of elephant (**Table 3.1**), with our sequence matching the forest clade and the old sequences in the GenBank database matching the savannah clade. A number of studies have indicated that the African savannah (*L. africana*) is a distinct species from the African forest elephant (*L. cyclotis;* Groves & Grubb 2000; Grubb et al. 2000; Roca et al. 2001; Roca et al. 2005; Roland et al. 2010*).* The barcoding COI sequence of our elephant specimen provided evidence that two different groups of elephant species geographically isolated were originally from Africa (99.56% match for *L. cyclotis*; 96.70% match for *L. africana)*. Because the blast search for our elephant barcode sequence showed perfect match for the forest than the savannah elephant in Genbank, we assumed that our elephant specimen originated in African forest (*L. cyclotis*).

We successfully obtained COI sequences for all dried blood, fresh, smoked, boiled and processed tissue samples including fresh human hair amplified with full barcode primers. The efficiency of newly designed primers by taxa category **(Figure 3.14)** and mini barcode primers for old and degraded products also confirmed the growing success of specific primer cocktails for vertebrates. Using the universal primers of Ivanova et al. (2006), Eaton et al. (2009) reliably amplified a 645bp fragment of the barcode regions for all mammal and reptile species. The level of sequence variation between individuals within a species was low. This clear sequence divergence between species, coupled with sequence conservation within species confirmed the barcode COI sequence as highly variable and

specific. We were also able to obtain barcode sequences from old hair samples collected from *Gorilla gorilla*, *Lemur catta*, *Panthera pardus,* and *Ailurus fulgens* skin amplified with mini barcode primers. However these sequences did not match perfectly in the GenBank database, which may be due to contamination. Indeed, the information about the preservation methods is often unknown, increasing the chance for contaminant DNA sequences to be targeted during PCR. However, fresh hair COI sequence matched perfectly *Homo sapiens* in the Genbank database.

Samples containing low levels of DNA are at risk of contamination from sources containing higher amount of the target DNA. We found old hairs to match domestic animals (i.e. bovid) in the databases possibly because samples from domestic animals were tested the day before in the same lab. Accordingly, Gilbert et al. (2005) reported that samples from species whose DNA might be reasonably expected to be present in the environment are of particular risk, especially those of the domestic animals. In order to avoid contamination when manipulating old hair samples, it is recommended to perform DNA extraction and pre-PCR in labs where no DNA extraction has been performed and no domestic animal's DNA has been studied before (Paboo 1989).

Since hairs are made up of keratin as fibrous structural protein we expected more DNA from hair follicle rather than the hair shaft. We obtained a lower amount of DNA from degraded hairs. However Higuchi and al. (1988) suggested DNA can be evenly extracted from the shaft hair. The utility of the mini primer pair despite its higher performance did not exclude the feasibility of extraction using the full barcode primers. The relevance of these primers depends on the specimen ages and the preservation methods. Interestingly, it has been shown that short DNA barcode sequences of about 120

base long- mini barcodes- can reliably distinguish species and can be used in old specimens with degraded DNA, where a full- length DNA barcode cannot be sequenced (Hajibabaei et al. 2007).

IV.2- Importance of species identification

Contributing in BOLD is very relevant for an efficient assessment of the wildlife resources firstly for species classification and for biodiversity research. We confirmed the existence of two elephant species in Africa colonizing two different habitats. Using the barcoding technology we could tell from which species of elephant belonged our specimens (**Table 3.1).** This case concerns morphologically different organisms as forest elephant seems to be smaller than the elephant of savannah. Using a species scientific name will be more reliable for taxonomic identification than the common name as many subspecies appears to be elevated to the species (Isaac et al. 2004). This might also help for vouchered collection during various field works.

We were also able to identify boiled, fresh pork meat and liver as *Sus scrofa*; as well as boiled and fresh beef as *Bos taurus*. In the humid tropic, it can be difficult to differentiate or identify some commercially harvested species as their meat is often partially degraded by the time they get to urban markets.

The five species (*Macropus giganteus*, *Crocuta crocuta, Tragelaphus strepsiceros, Hippotragus niger* and *Loxodonta cyclotis*) sequences added to BOLD and Genbank databases showed high genetic variation with the closest related species. The contribution to database requires species references and catalogue numbers.

The sable antelope's barcode sequence match could not be found in BOLD and the nearest match in GenBank was *Capricornis sumatraensis* (88%). Such value was expected

for the discrimination of the two herbivores species that are actually geographically isolated (sable antelope is originally from Africa while the *capricornis sumatraensis* is from Asia).As seen in the phylogenetic tree (**Figure 3.9)**, Zebra2 and Elephant1 are not at their expected position, most likely because both samples were contaminated by DNA from Caracal lynx, hence their proximity to Caracal lynx despite being from different families. It also appears from the tree that species are gathered by category but at random positions. For example, Artiodactyls' species appeared before kangaroos and after proboscidae. So, this phylogenetic tree broadens our understanding for the effectiveness of the species distribution after 500 replications by random sampling.

Looking at the phylogenetic tree **(Figure 3.22)**, regardless of the storage methods and the type of specimen, most of the closely related species are gathered together. This explained why Deer, a Cervidae, appeared among Bovidae species. Therefore, DNA sequences can confidently tell us the source of many unknown and mislabeled products. By allowing easy and rapid identification of animal products, DNA barcoding technology can help slowing down the global rate of the bushmeat trade. Via this tool, conservation organizations can identify the origin and the identity of animal products seized at the bushmeat markets or airports, as well as animal species available at many Zoos in the world.

IV.3- Conservation strategy

The success of the legal trade in animal products will depend on the ability to identify to which species the specimen belongs and how much these legally traded products could affect the wildlife population of the area. The utility of molecular tools in the field of ecology are slowing down the wildlife and wildlife products traffic in the world.

Recent studies have confirmed that Cheetahs (*Acinonys jubatus)* and African wild dogs *(Lycaon pictus*) have become essentially extinct in Cameroon. A three year study by the Institute of Environmental Sciences at Leiden University in Netherlands found that the same factor that push cheetahs and African wild dogs to local extinction, have also left Cameroon's other big predators hanging by a thread including lion, leopard, and two species of hyena: the spotted and the stripped hyenas (Hance 2010). The bushmeat problem could be solved in the tropics if the local governments in collaboration with other international conservation agencies established a strategy for local people's awareness and the development of farming practice. For example, the promotion of game farming and breeding of domestic species such as poultry and fish might help to meet the demand for meat (Mbete et al. 2011). Providing alternative sources of protein to replace wild meat would indeed be an efficient way to keep some people away from illegal animal harvest.

Chapter V: General conclusion.

V.1 - Conclusion

The DNA barcoding initiative has proved to be a valuable tool for efficient species identification. DNA barcodes were used to test all specimens from the Zoos; the dried blood samples obtained were not actually distinguishable without specimens' name and reference numbers. Among blood samples from different specimens collected on the FTA cards and stored at the room temperature, only Guinean pig blood failed for sequencing. So the effectiveness of this preservation method without the need of refrigeration during many field works was confirmed. The FTA cards technology is economically important as samples can easily be transported around the world. DNA samples preserved over several months on FTA cards were successfully sequenced and the species origin identified.

Result from the radial tree confirmed the utilization of DNA barcoding for the species level variation as the extinct mammoth appeared to be closer to African elephants than the Asian elephant while according to evolutionary theory both African and Asian elephants descended from mammoth. Overall we were able to successfully add new sequence records to the BOLD database system. We realized that old tissue samples (hairs or skin) amplified with mini primers need to be investigated in labs where no domestic meat DNA has been manipulated. Regardless of the type of products, this technique had proven to be useful in species identification at low cost.

V.2 - Management implications (Case study of Cameroon)

Tropical biodiversity is threatened by overexploitation; enhanced by the illegal trade of wildlife products. The identification of the species involved has proven difficult, hence the efforts of the CITES to control the trade. Cameroon is one of the most diverse countries in the world in term of fauna and flora, but is experiencing a high level of illegal trade. Fifty six percent of specimens found in Toronto and Granby Zoos occur in Cameroon and Ghana using the checklist of mammal species being used in bushmeat trade, and found that there was no significant difference between mammal status and category (whether bushmeat or not) in Cameroon **Figure 4.1.** Recent investigations in Brazzaville (Congo) suggested that 88.3% of the surveyed households consumed bushmeat, mostly Artiodactyls species (48.5%). Most of these large animals are at risk and classified in the IUCN red list as endangered species. The lack of law enforcement and corruption lead to the growing bushmeat market in the region, often with uncertainty on the real identity of species involved. One has to be an expert to differentiate wild from domestic meat, both in butcheries or at the international airports, when we cannot morphologically recognize meat on sale. Monitoring illegal bushmeat trade and enforcing wildlife regulations have proven difficult, because it is often impossible to determine the species of origin of many animals' products such as processed filets, hides, and bones. The identification of these products is essential for the evaluation of illegal bushmeat trade.

References

Albrechtsen, L., Macdonald, D.W., Johnson, P.J., Castelo, R. and Fa, J.E. 2007. Faunal lost from bushmeat hunting: empirical evidence and policy implications in Bioko Island. *Environmental Science Policy* 10: 654-667.

Alves, R.R.N. and Rosa, I.L. 2005. Why study the use of animal products in traditional medicines? *Journal of Ethnobiology and Ethnomedicine* 1:1- 5.

Arlet, M.E., and Molleman, F. 2007. Rodents damage crops more than wildlife in subsistence agriculture on the northern periphery of Dja Reserve, Cameroon. *International Journal of Pest Management* 53: 237-243.

Avise, J.C. 2000. Phylogeography: The history and formation of species. *Havard University Press*, Cambridge, MA: 447p.

Bennett Hennessey, A. 1995. A study of a meat trade in Ouesso, Republic of Congo. Wildlife Conservation Society, Bronx, New York: 19p.

Bennett, E.L., Eves, H.E. and Robinson, J.G. 2002. Why is eating bushmeat a biodiversity crisis? *Conservation in Practice* 3: 28-29.

Bowen-Jones, E. and Pendry S. 1999. The threat to primate and other mammals from the bushmeat trade in Africa and how this threat could be diminished. *Oryx* 33: 233-246.

Brandt, A.L., Ishida, Y. Georgiadis, N.J., and Roca, A.L. 2012. Forest elephant mitochondrial genomes reveal that elephantid diversification in Africa tracked climate transitions. *Molecular Ecology.* 21: 1175-1189.

Brown, D. 2003. Is the best the enemy of the good? Livelihoods perspective on bushmeat harvesting and trade – some issues and challenges. *Paper presented at the International Conference on Rural Livelihoods, Forest and Biodiversity,* London: 20p.

Brownlee, C. 2004. DNA Barcodes: Life under the scanner. *Science News* 166: 360p.

Chapman, C.A., Michael, J.L., and Harriet, A.C. 2006. What hope for African Primate Diversity? *African Journal of Ecology* 44: 116- 33.

Daniel, S. and Esmond, M. 2009. The USA's ivory markets- How much a threat to elephant? *Pachiderm* 45: 67-76.

Drummond, A.J., Ashton, B., Cheung, M., Heled, J., Kearse, M., Moir, R., Stones-Havas, S., Thierer, T. and Wilson, A. 2009. Geneious Version 4.8 Available from http://www.geneious.com/.

Eaton, M.J., Martin, A., Thorbjarnarson, J., and Amato G. 2009. Species –level diversification of African dwarf crocodiles: (genus *Osteolanus*): a geographic and phylogenetic prospective. *Molecular Phylogenic Evolution* 50: 496-506.

Eaton, M.J., Meyers, G.L., Kolokotronis, S.O., Leslie, M.S., Martin, A.P., Amato, G. 2010. Barcoding bushmeat: molecular identification of Central African and South American harvested vertebrates. *Conservation Genetic* 11: 1389-1404

Emslie, R., and Brooks, M. 1999. African Rhino status survey and conservation action plan. *Gland and Cambridge: IUCN /SSC* African Rhino Specialist Group: 92p.

Gilbert, H.J., Bandelt, M., Hofreiter, M. and Barnes, I. 2005. Assessing ancients DNA studies. *Trends Ecology and Evolution* 20: 541-544.

Groves, C.P. and Grubb, P. 2000. Do *Loxodonta cyclotis* and *L. Africana* interbreed? *Elephant* 2: 4-7.

Grubb, P., Groves, C.P., Dudley, J.P., and Shoshani, J. 2000. Living African elephants belong to two species: *Loxodonta africana* (Blumenbach, 1797) and *Loxodonta cyclotis* (Matschie, 1900). *Elephant* 2:1-4.

Hance, J. 2011. Over 80% of urban Congolese eat bushmeat. *Environmental News.* Mongabey.com.

Hance, J. 2010. Cameroon says goodbye to cheetahs and African wild dog. *Environmental News*. Mangabey.com.

Hajibabaei, M., Singer, G.A.C., Hebert, P. D.N. and Hickey, D. 2007. DNA barcoding: how it complements taxonomy, molecular phylogenics and population genetics. *Trends in Genetics* 23: 162-172.

Hebert, P.D.N., Cywinska, A., Ball, S.L., and deWaard, J.R. (2003a). Biological identifications through DNA barcodes. *Proceedings of the Royal Society London - Biological Science* 270: 313-321.

Hebert, P.D.N., Ratnasingham, S. and deWaard, J.R.. 2003b. Barcoding animal life: cytochrome c oxidase subunit I divergence among closely related species. *Proceedings of the Royal Society London*. -*Biological Science* 270: S96-S99.

Higuchi, R., von Beroldingen C.H., Sensabaugh, G.F. and Erlich, H.A. 1988. DNA typing from single hairs. *Letters to Nature* 332: 543-546.

IUCN, 2010. IUCN Red List of Threatened Species. 2010.4. < www.iucnredlist.org > Download on 17 May 2010.

Isaac, N.J.B., Mallet, J., and Mace, G.M. 2004. Taxonomic inflation: its influence on macro ecology and conservation. *Trends in Ecology and Evolution* 9: 464-469.

Ivanova, N.V., deWaard, J.R., and Hebert, P.D.N. 2006. An inexpensive automatisation-friendly protocol for recovering high quality DNA. *Molecular Ecology Notes* 6: 998-1002.

Kohn, A.E., and Eves, H.E. 2006. The African Bushmeat Crisis: A Case for Global Partnership. Berkeley Electronic Press Legal Series. Paper 1758, 24pp.

Mbete, A.R., Mboko, H.B., Racey, P., Ntsakala, A.M., Nganga, I., Vermelen, C., Doucet, J.L., Hornick, J.L. and Leroy, P. 2011. Household bushmeat consumption in Brazzaville, the Republic of the Congo. *Tropical Conservation Science* 4: 187-202.

Meintjes, P., Duran, C., Kearse, M., Moir, R., Wilson, A., Havas, S.S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Thierer, T., Asthon, B. and Heled, J. 2012. Geneious basic: An intergrated and extendable desktop software

platform for the organization and analysis of sequence data. *Bioinformatic Advance Access*: doi:10.1093/bioinformatics/bts199.

Meusnier, I., Singer, A.C., Landry, J.F., Hickey, D.A., Hebert, P.D.N. and Hajibabaei, M. 2008. A universal DNA mini barcode for biodiversity analysis. *Biological Medicine Central Genomic* 9: 214.

Milner-Gulland, E.J. and Bennett, E.L. 2003. Wild meat: the bigger picture. *Ecology and Evolution* 18:351-357.

MINEF. 1998. List of animal per category. Working document of the Ministry of Wildlife and Forestry, Government of Cameroon, report, 4p.

Paboo, S. 1989. Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. *Proceedings of the National Academy of Sciences*. *USA* 86: 1939-1943.

Ratnasingham, S. and Hebert, P.D.N. 2007. BOLD: The barcode of life data system. *Molecular Ecology Notes* 7:355-364.

Redford, K.H. 1992. The empty forest. *BioScience* 42: 412-422.

Roca, A.L., Georgiadis, N., and O' Brien, S.J. 2001. Genetic evidence for two species of elephant in Africa. *Science* 293: 1473-1477.

Roca, A.L., georgiadis, N., and O'Brien, S.J. 2005. Cytonuclear genomic dissociation in African elephant species. *Nature Genetics* 37: 96-100.

Rohland, N., Reich, D., Mallick, S., Meyer, M., Green, R.E., Georgiadis, N.J., Roca, A.L., and Hofreiter, M. 2010. Genomic DNA sequences from mastodon and woolly mammoth reveal deep speciation of forest and savannah elephant. *PloS Biology* 8**:** e1000564.

Saitou, N. and Nei, M. 1987. The neighbor- Joining method: A new method for reconstructing Phylogenetic Trees. *Molecular Biology and Evolution* 4: 406-425.

Smith, L.M. and Burgoyne, L.A. 2004. Collecting, archiving and processing DNA from wildlife samples using FTA® databasing paper. *BioMed Central Ecology* 4: 4.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software. Version 4.0. *Molecular Biology and Evolution* 24: 1596-1599.

Vuissoz, A., Worobey, M., Odegard, N., Bunce, M., Machado, C.A., Lynnerup, N., Peacock, E.E., Thomas, M. and Gilbert, P. 2007. The survival of PCR amplifiable DNA in cow leather. *Journal of Archaeology* 34: 823-829.

Ward R.D., Zemlak, T.S., Innes, B.H., Last, P.R. and Hebert, P.D.N. 2005. DNA barcoding Australia's fish species.*Philosophical Transactions of the Royal Society of London. Series B, Biological sciences* 360: 1847-1858.

Wasser, K.S., Clark, W.J., Drori, O., Kisamo, E.S., Mailand, C., Mutayoba, B., and Stephens, M. 2008. Combating the Illegal Trade in African Elephant Ivory with DNA Forensics. *Conservation Biology* 22: 1065-1071.

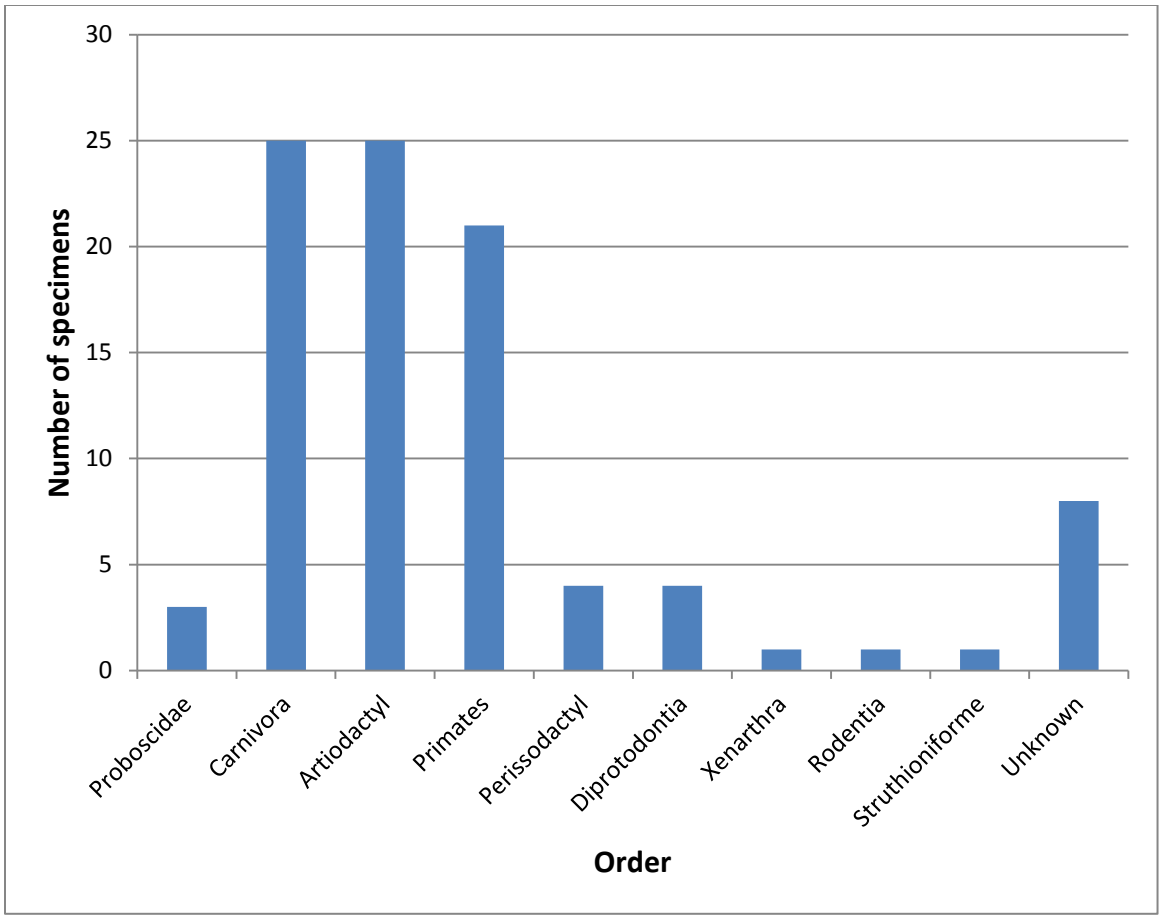


Figure 2.1: Number of specimens collected per animal order. Unknown stands for unidentified specimens.

Forward primers

- Vf1 : 5'-TTCTCAACCAACCACAAAGACATTGG-3'
- Vf1d : 5'-TTCTCAACCAACCACAARGAYATYGG-3'
- Vf1i: 5'-TTCTCAACCAACCAIAIGAIATIGG-3'

Reverse primers

- Vr1 : 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'
- Vr1d: 5'-TAGACTTCTGGGTGGCCRAARAAYCA-3'
- Vr1i : 5'-TAGACTTCTGGGTGICCIAAIAAICA-3'

Figure 2.2: The universal primers for vertebrates (Ivanova et al. 2006). Vfi and Vri stand for primer forward and reverse with inosine respectively: V for vertebrate; f for forward; r for reverse; d for degenerate (i.e. containing R and Y); and i for inosine.

Forward Primers

- Vf1d : 5'- TTCTCAACCAACCACAARGAYATYGG - 3'
- Vf1d Artiodactyls : 5'- TACTCAACAAACCAYAARGAYATYGG – 3'
- Vf1d Carnivores : 5'- TTTTCAACYAATCACAARGATATTGG – 3'
- Vf1d Primates : 5'- TTCTCDACDAACCAYAAAGAYATTGG – 3

Reverse Primers

- Vr1d : 5'-TAGACTTCTGGGTGGCCRAARAAYCA - 3'
- Vr1d Artiodactyls : 5'-TATACTTCRGGGTGYCCRAARAAYCA - 3'
- Vr1d Carnivores : 5'-TARACTTCTGGRTGRCCRAARAATCA - 3'
- Vr1d Primates : 5-TARACTTCRGGGTGNCCRAARAATCA - 3'

Figure 2.3: New primers for specific vertebrate groups

Vf1d and Vr1d represent the mammalian primers developed by Ivanova et al. (2006). These are compared for specific primer pairs developed for artiodactyls, carnivores, and primates. The highlighted sites represent the more expressed nucleotides during alignment in Geneious: R (A or G); Y (T or C); D (A or T or G); N (A or T or C or G). The blue bars represent the COI gene and the barcode region respectively.

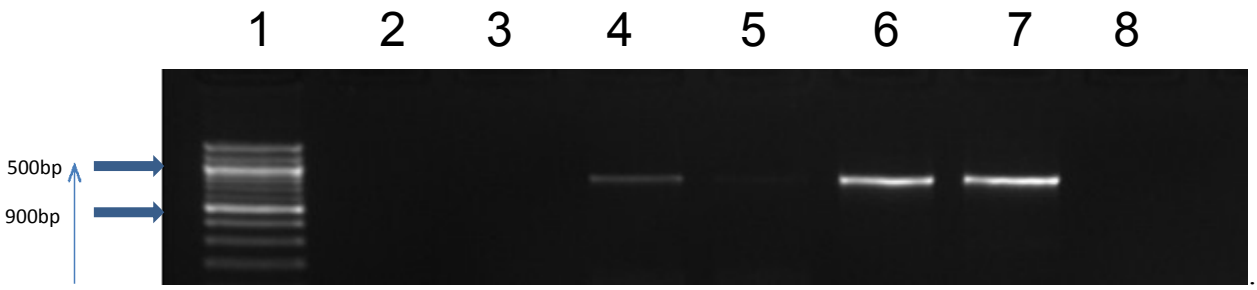


Figure 3.1: PCR amplification results for extraction methods tested on dried tissue samples preserved on the FTA card after several weeks. Well 1: Molecular size marker; Well 2: blank; Well 3: blank; Well 4: FTA purification reagent; Well 5: room temperature PH treatment; Well 6: Nucleospin; Well 7: Qiagen; Well 8 is the negative control. The 500bp (top) and the 900bp (bottom) size marker bands are shown at the left.

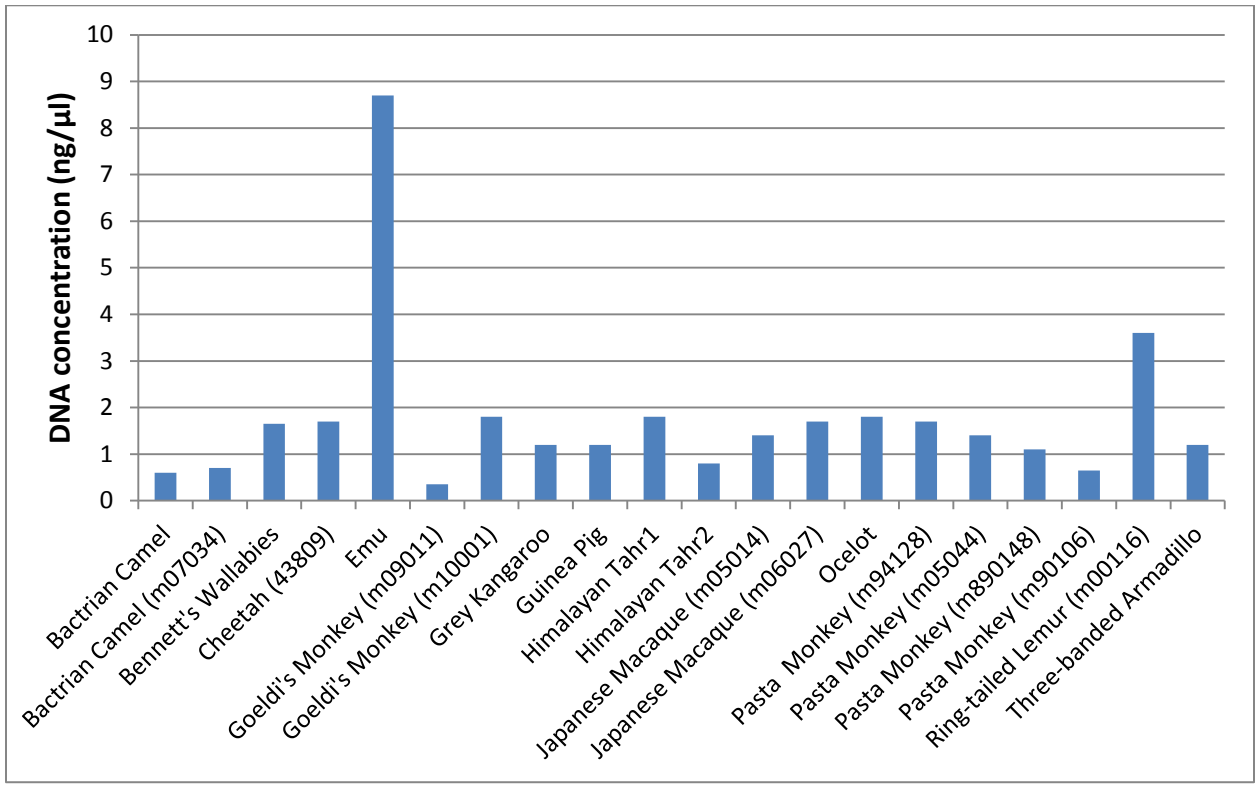


Figure 3.2: DNA concentration (ng/ul) from 50ul stored on FTA cards blood samples from 19 species of mammals and 1 bird. The species names are listed alphabetically.

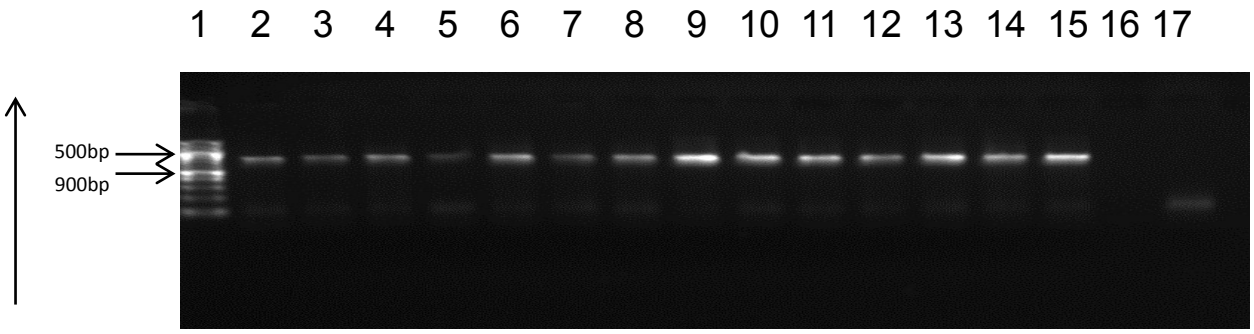


Figure 3.3: PCR amplification results for 15 dried blood samples from animals at the Toronto Zoo. Well 1: molecular size marker; Wells 2-15: dried blood samples from Toronto Zoo; Well 16: blank; Well 17 is the negative control. The 500bp (top) and the 900bp (bottom) size marker bands are shown on the left side.

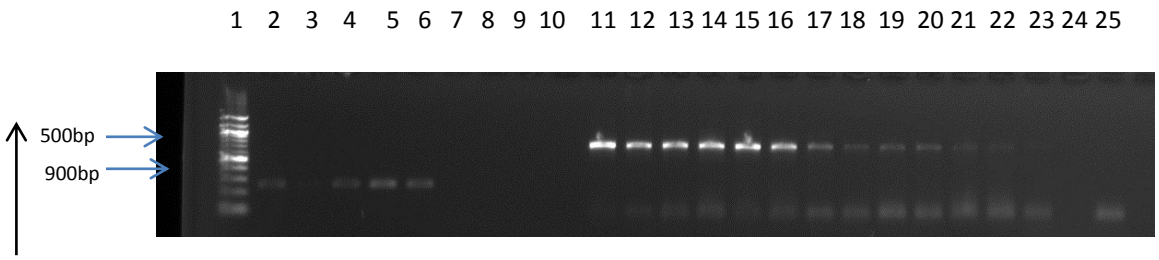


Figure 3.4: PCR amplification results for 13 dried blood samples obtained from Granby Zoo. Well 1: molecular size marker; Wells 2-10: old hair samples; Wells 11- 23: dried blood samples from Granby Zoo; Well 24 is empty and Well 25 is the negative control**.** The 500bp (top) and the 900bp (bottom) size marker bands are shown on the left side.

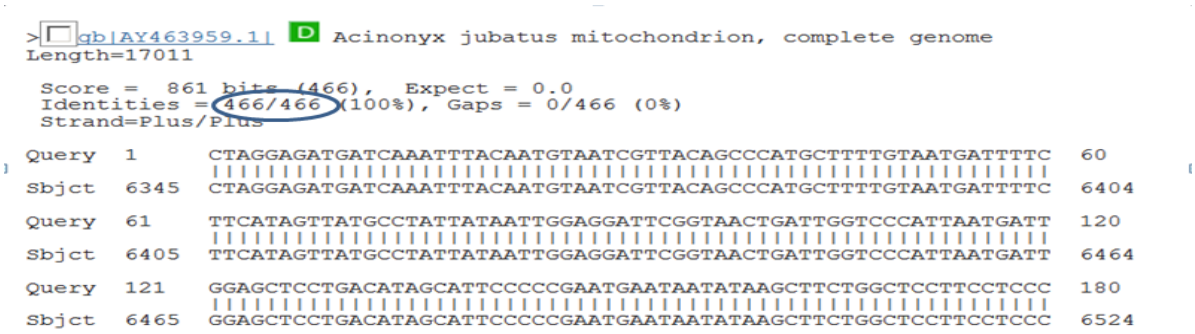


Figure 3.5: Comparison of cheetah barcode sequence with the reference sequence in Genbank.

Our query matches perfectly the sequence of the GenBank (subject).

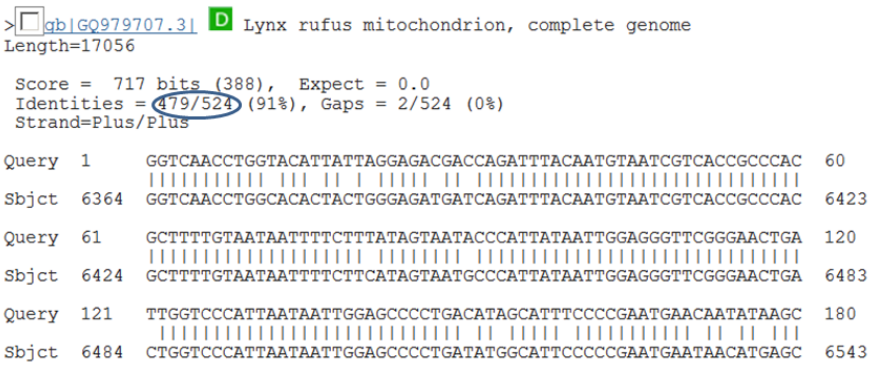
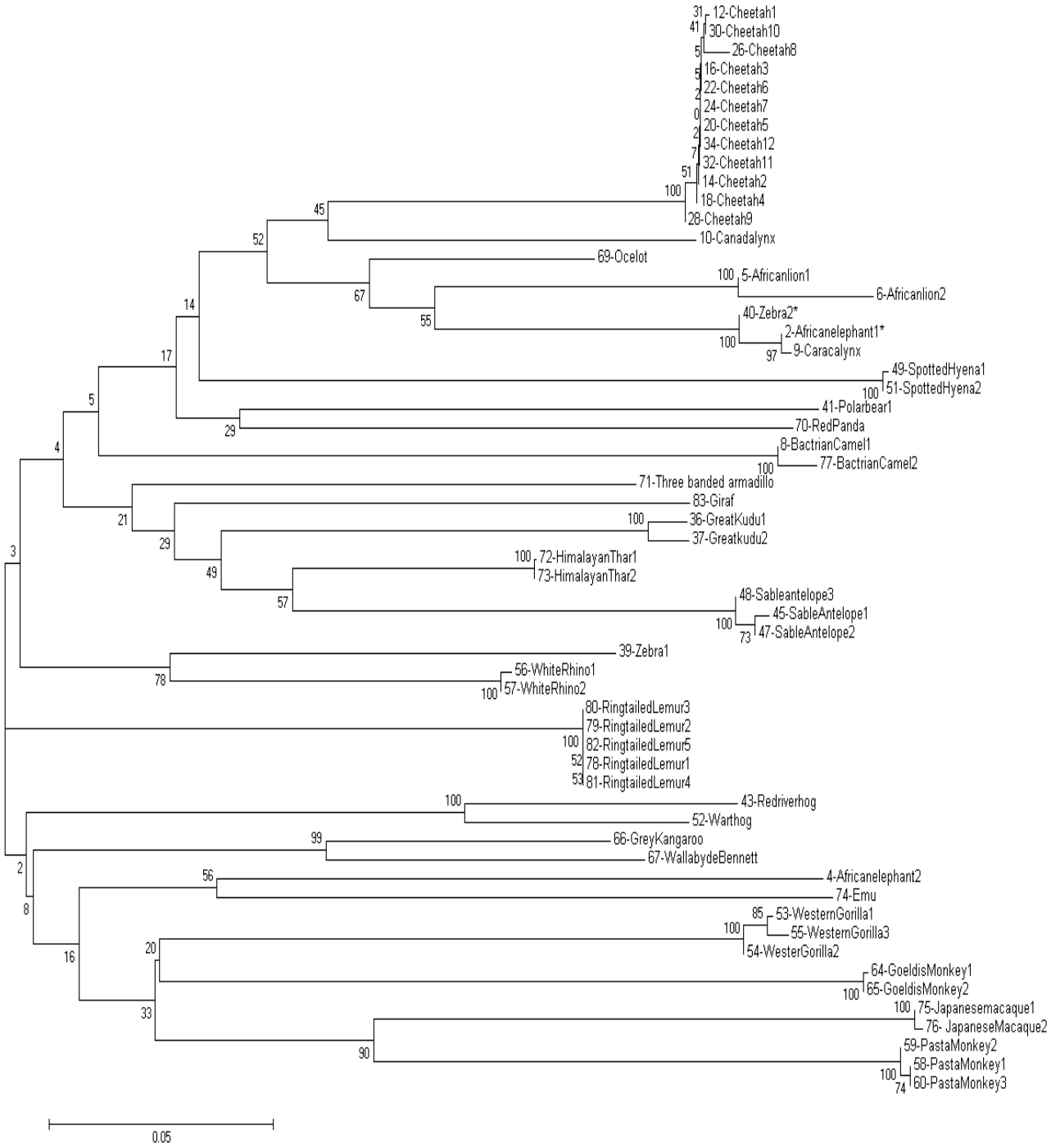


Figure 3.6: Comparison of barcode sequences of related species (*Lynx canadensis vs. Lynx rufus*) from North America. There are 45 nucleotide differences between the query and the subject; this represents (9%) of sequence dissimilarity.



Contaminated specimen (*)

Figure 3.7: Phylogenetic tree showing the relationship between the barcode Cytochrome Oxidase I (COI) gene sequences of various taxonomically verified specimens. Scale bar represents genetic distance. Numbers on the nodes are bootstrap values after 500 replications and the numbers linked to different specimens are DNA sequence references.

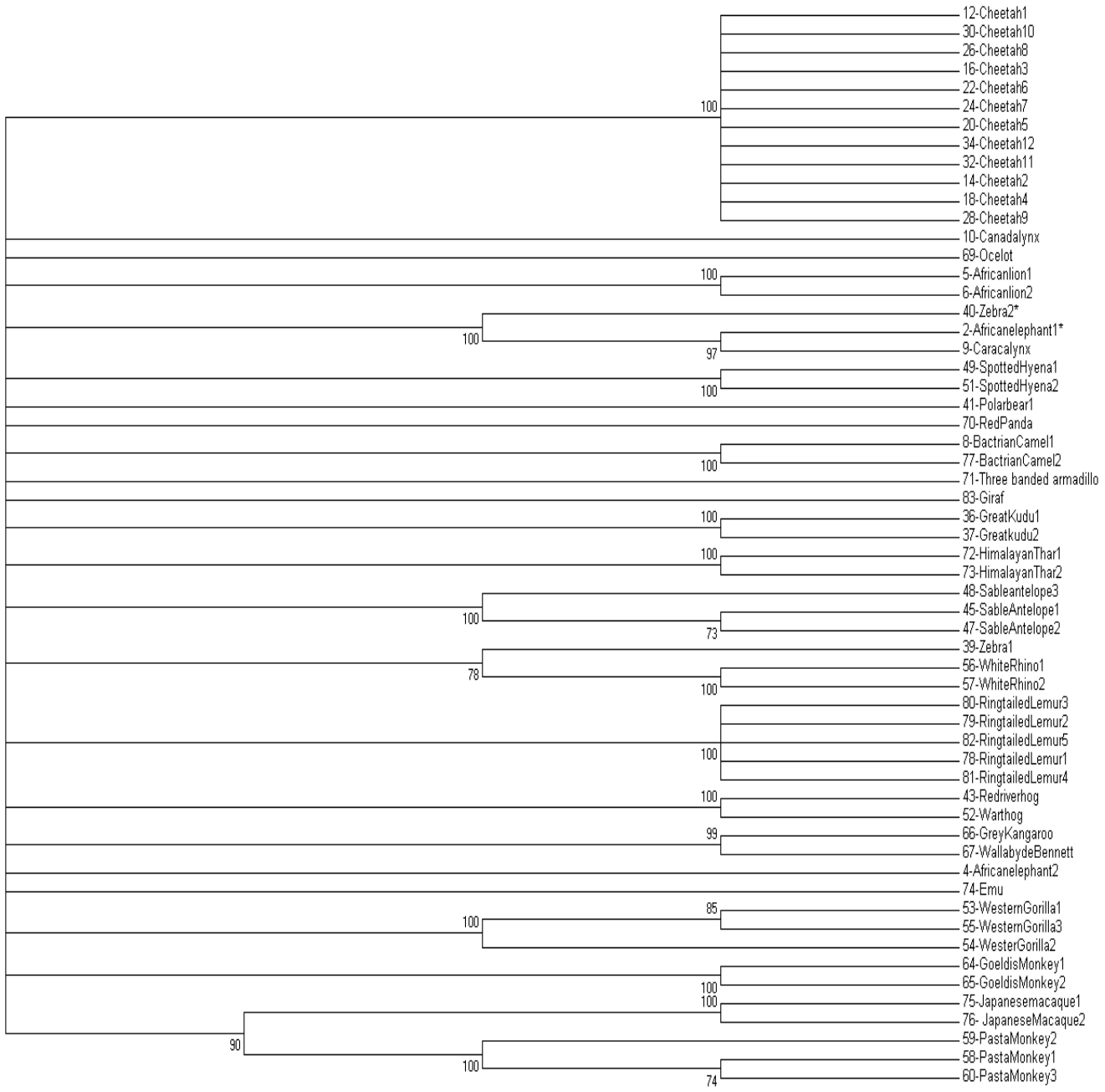


Figure 3.8: The same tree as shown in Figure 3.7, but with all nodes with low bootstrap support collapsed. This illustrates that groupings at greater phylogenetic distances are not well supported.

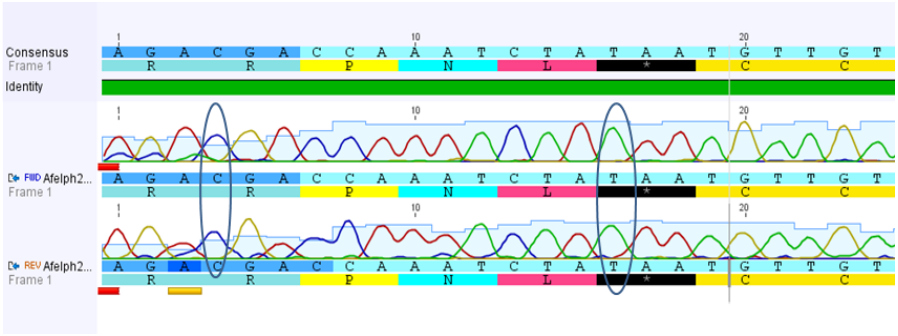


Figure 3.9: Editing sequence of African elephant2 forward and reverse alignments using Geneious software

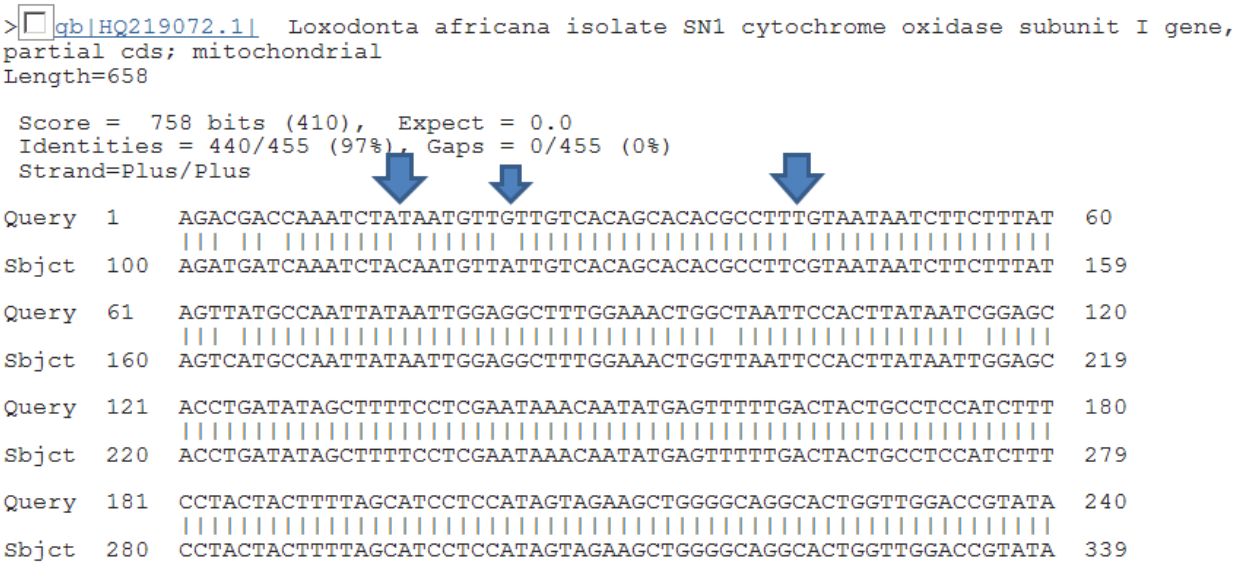


Figure 3.10: BLAST search for an African elephant specimen (labeled as Africanelephant2) obtained from the Zoo and compared with sequences from the same species in GenBank, the percent identities between our query (Africanelephant2) and the subject (*Loxodonta africana)* are 97% (15 nucleotides difference) though both directions are realized; the arrows show some blank spaces at positions 16, 23 and 43; also no gaps was found.

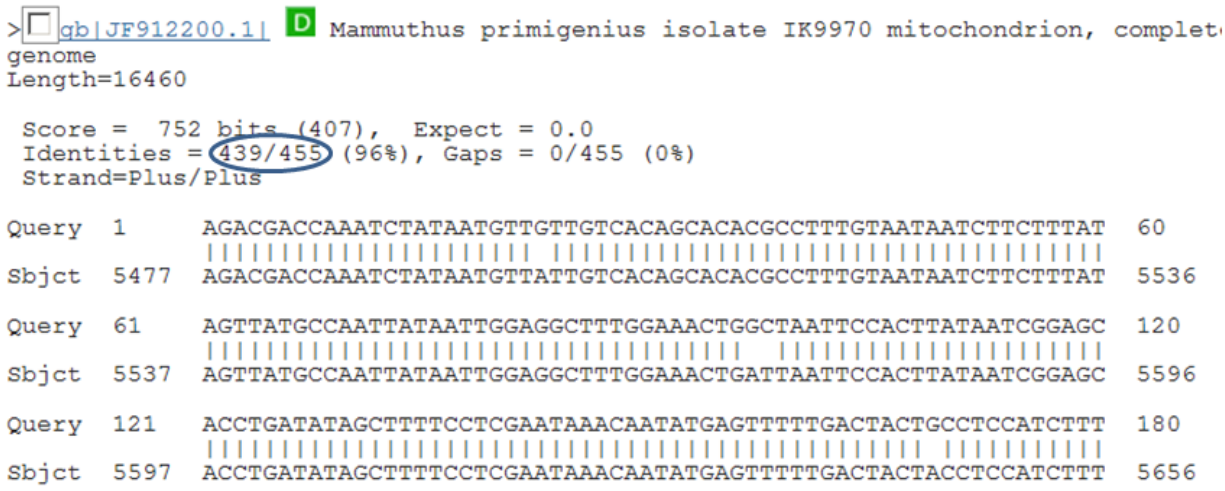


Figure 3.11: Result of BLAST search for Africanelephant2 compared *Mammuthus primigenuis*; 16 nucleotides difference between both species; no gaps found in both sequences (0/455); also both directions (query and subject) are realized; no gaps was found.

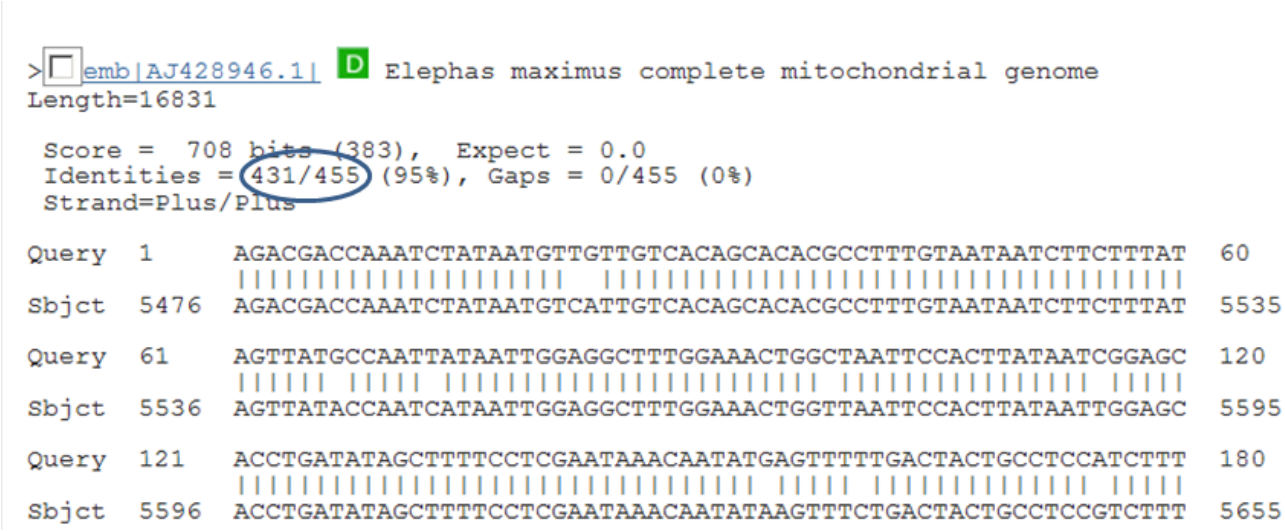


Figure 3.12: Result of BLAST search for Africanelephant2 compared with *Elephas maximus;* 14 nucleotide divergences between both species with 0 gap; both directions are realized so a nucleotide from the query sequence matches the respective complementary nucleotide of the subject.

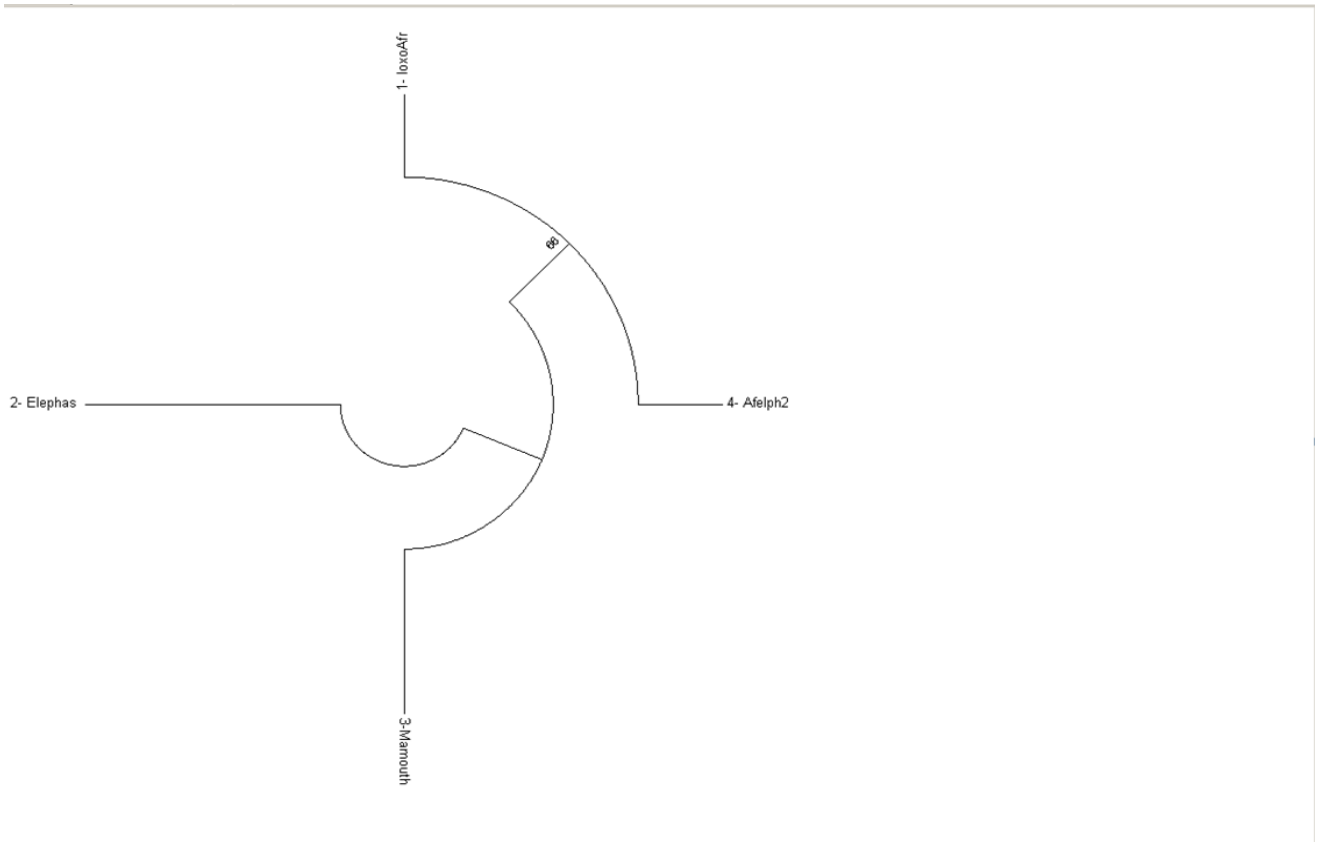


Figure 3.13: Radial NJ tree showing the evolutionary relationship between elephant species based on the single mtCOI as marker. 1- loxoafr stands for *Loxodonta africana*; 2-Elephas stands for *Elephas maximus*; 3- Mamouth is *Mammuthus primigenius and* 4- Afelph2 stands for Elephant DNA barcode.

- Vf1d artiodactyls : 5’- TACTCAACAAACCAYAARGAYATYGG – 3’
- Vr1d artiodactyls : 5’-TATACTTCRGGGTGYCCRAARAAYCA - 3’

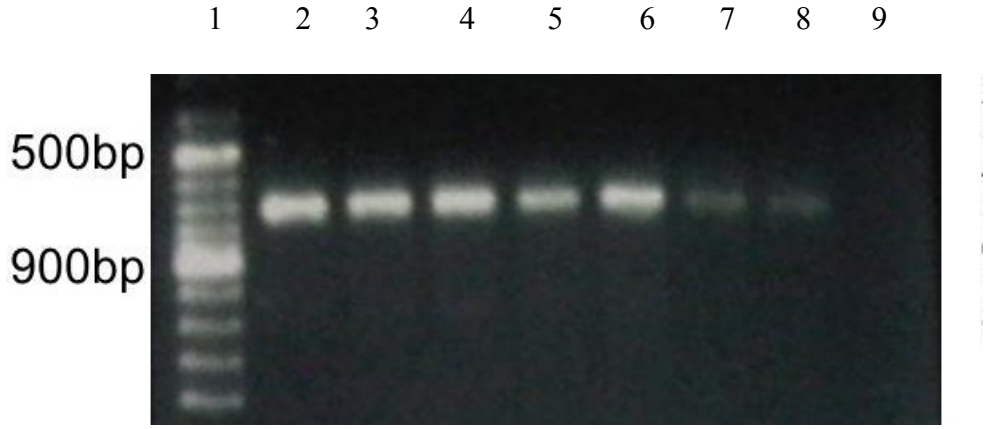


Figure 3.14: PCR amplification results for various artiodactyls samples amplified with the specific primer pair for artiodactyls (the targeted region is between 500 and 900bp). Well 1: molecular size marker; Well 2: cooked pork; Well 3: frozen pork; Well 4: pork liver; Well 5: calf liver; Well 6: frozen beef; Well 7: fresh beef; Well 8: grilled beef; Well 9 is the negative control (contamination control).

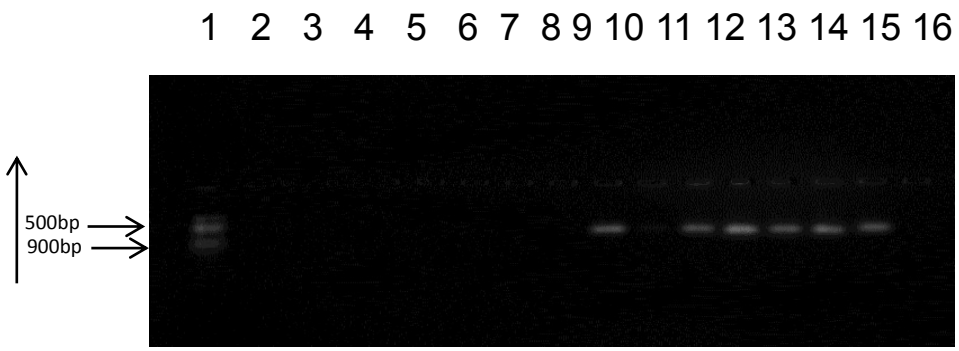


Figure 3.15: PCR amplification results for various field samples (e.g. old hair, smoked meat). The numbers above the gel photgraph label the various DNA samples as follows. Well 1: molecular size marker; Wells 2 - 8: old hair samples; Well 9: fresh hair; Well 10: blank; Wells 11, 12, 13: Jambon de campagne, Proscuito parma, Keiser fleisch, respectively; Well 14: hamburger; Well 15: processed meat (sausage); Well 16 is the negative control. The 500bp (top) and the 900bp (bottom) size marker bands are shown on the left side.

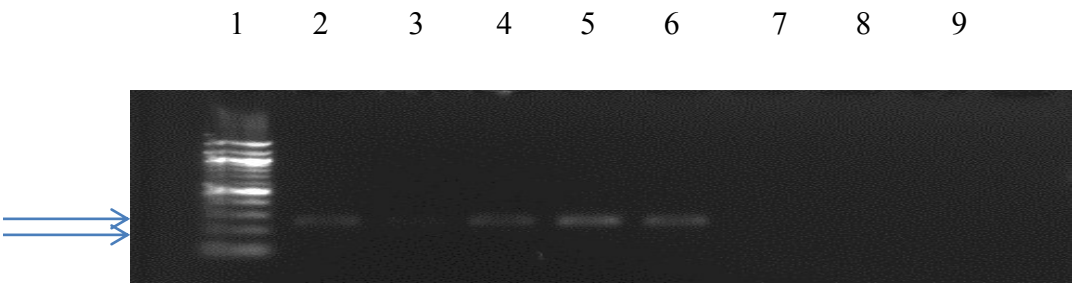


Figure 3.16: Mini barcode amplification results for old hair samples using Aquaf2 forward / mammals cocktail primers. Well 1: molecular size marker; Well 2: regular gorilla DNA; Well 3: blank; Well 4: regular giant panda DNA; Well 5: regular ring-tailed lemur DNA; Well 6: regular leopard DNA; Well 7: 1/100 diluted gorilla DNA; Well 8: 1/100 diluted ring-tailed lemur DNA; Well 9: 1/100 diluted giant panda DNA. All diluted DNA stock failed. The arrows represent the barcoding region from the bottom (200bp) to the top (300bp), the direction of electrophoretic migration is from the top to the bottom.

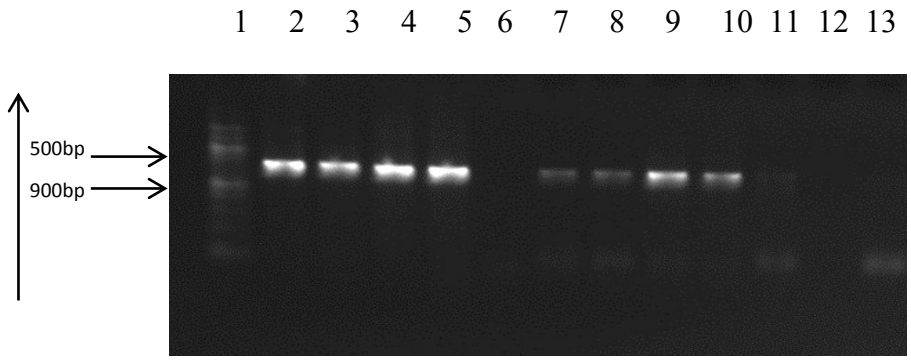
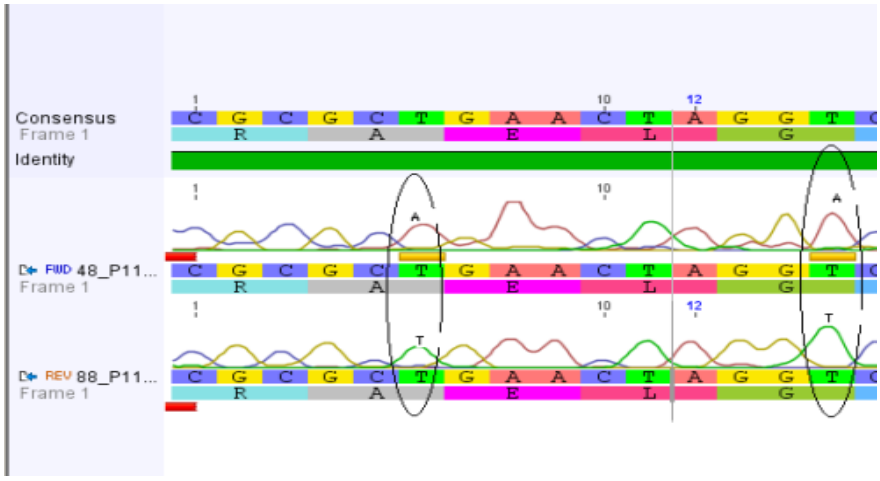


Figure 3.17: Full barcode amplification results of unknown meat samples from restaurants. Well 1: molecular size marker; Well 2: dried meat #1; Well 3: dried meat #2; Well 4: dried meat #3; Well 5: dried meat #4; Well 6: meat juice A; Well 7: meat juice B; Well 8: meat juice C; Well 9: meat juice D; Well 10: positive control; Well 11: blank; Well 12: blank; Well 13 is the negative control. Specimen 6 (meat juice) failed to amplify.




```
>gb|GU261717.1| Gallus gallus isolate YP20123 breed Chigulu mitochondrion, complete
genome
Length=16784

 Score =  832 bits (450),  Expect = 0.0
 Identities = 492/513 (96%), Gaps = 0/513 (0%)
 Strand=Plus/Plus

Query  1     CGCGCTGAACTAGGTCAGCCCGGAACCCTATTAGGAGATGATCAAATTTACAATGTAATC  60
             ||||| |||||||| |||||||||| || |||||||| ||||||||||||||||||||||
Sbjct  6764  CGCGCAGAACTAGGACAGCCCGGAACTCTCTTAGGAGACGATCAAATTTACAATGTAATC  6823

Query  61    GTCACAGCCCATGCTTTCGTCATAATCTTCTTTATAGTTATACCCATTATGATCGGGGGT  120
             |||||||||||||||||||||||||||||||||||||||||||||| |||||||| ||
Sbjct  6824  GTCACAGCCCATGCTTTCGTCATAATCTTCTTTATAGTTATACCCATCATGATCGGTGGC  6883

Query  121   TTCGGAAACTGACTAGTCCCGCTTATAATCGGTGCCCCAGACATAGCATTCCCCCGCATA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6884  TTCGGAAACTGACTAGTCCCGCTTATAATCGGTGCCCCAGACATAGCATTCCCCCGCATA  6943
```

Figure 3.18: Pair wise alignments of boiled chicken sequence in Geneious (upper panel) and comparison of the corresponding sequence region in Genbank (lower panel). A species level match was not found (21 nucleotides difference; this represents 4% of divergence). Both chromatograms show different nucleotides at positions 6, 15 (as shown the arrows). The complementary positions from the subject in GenBank are 6769, 6778.

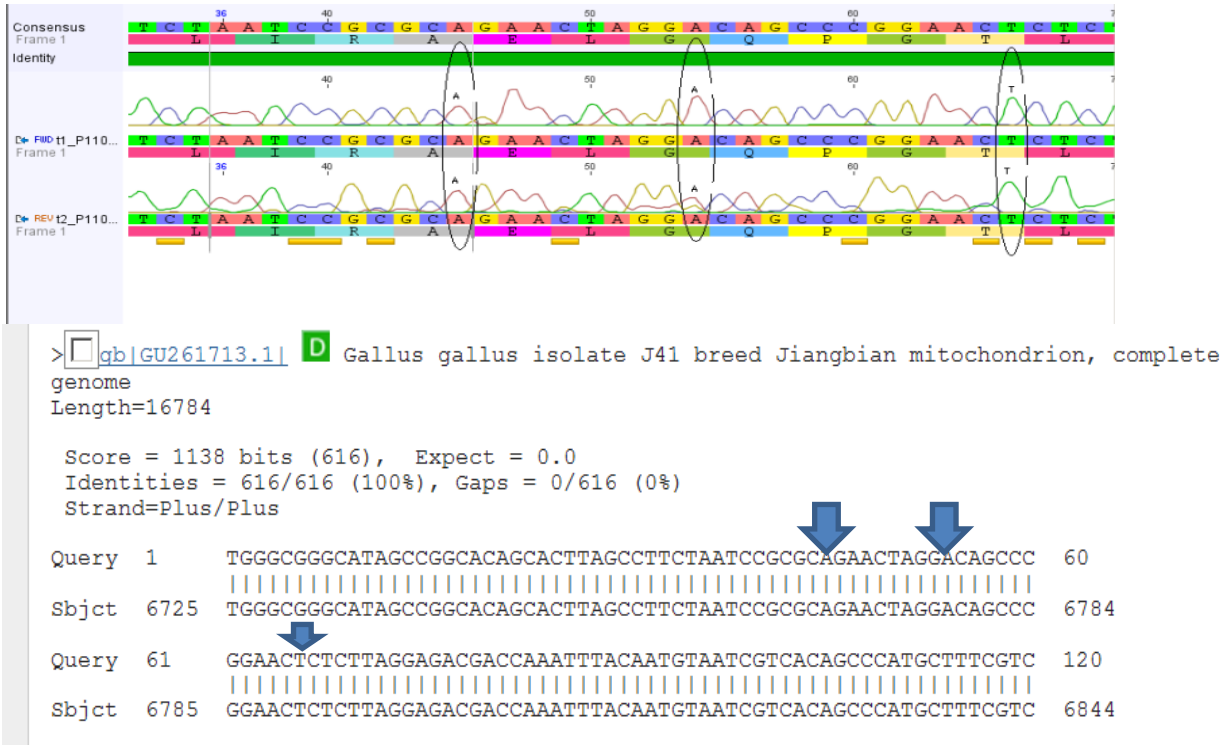


Figure 3.19: Pair wise alignments of fresh chicken sequence in Geneious (upper panel) and comparison to find the corresponding sequence match in Genbank (lower panel). The corresponding positions from the subject are similar as those of boiled chicken (6769, 6778 and 6790).

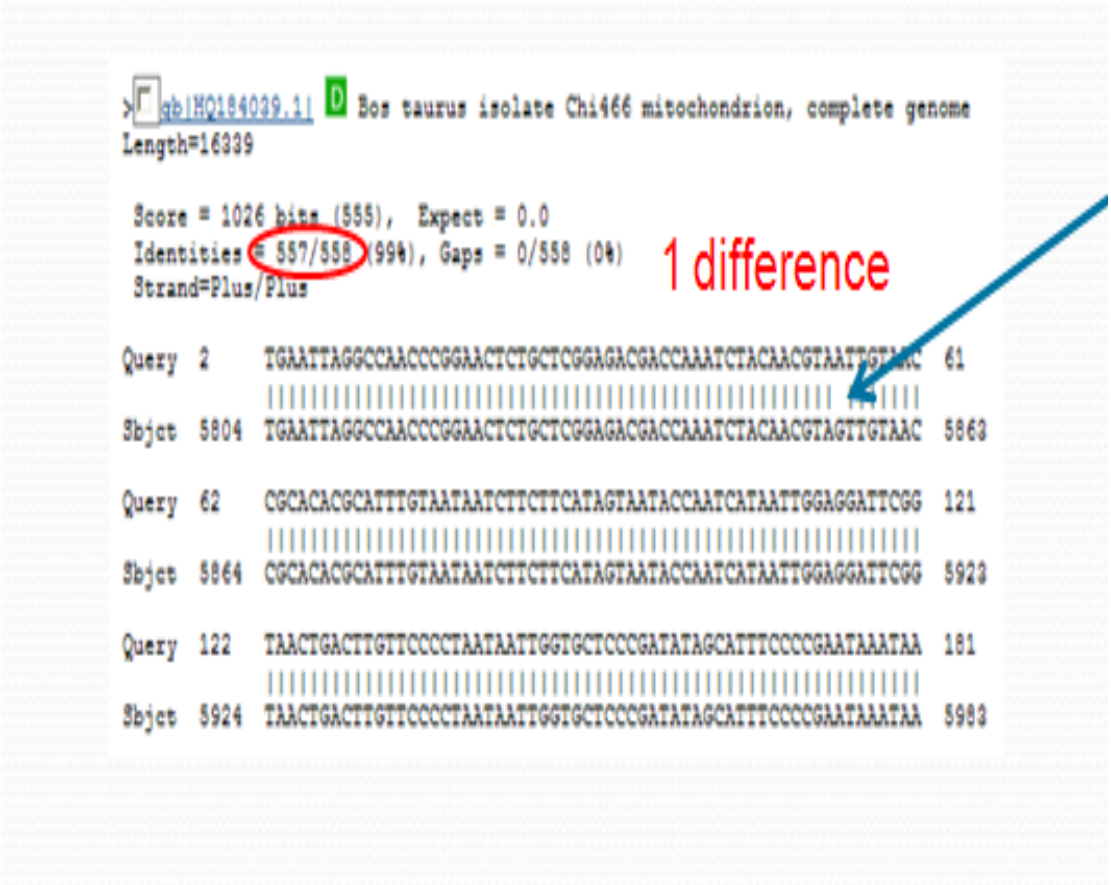


Figure 3.20: Database search for a different beef sample; the comparison of our boiled beef with the database match for *Bos taurus* as expected. The percent identities is 99%, the difference appears at the position 53 (A instead of G). The observed difference is not a sequencing error.

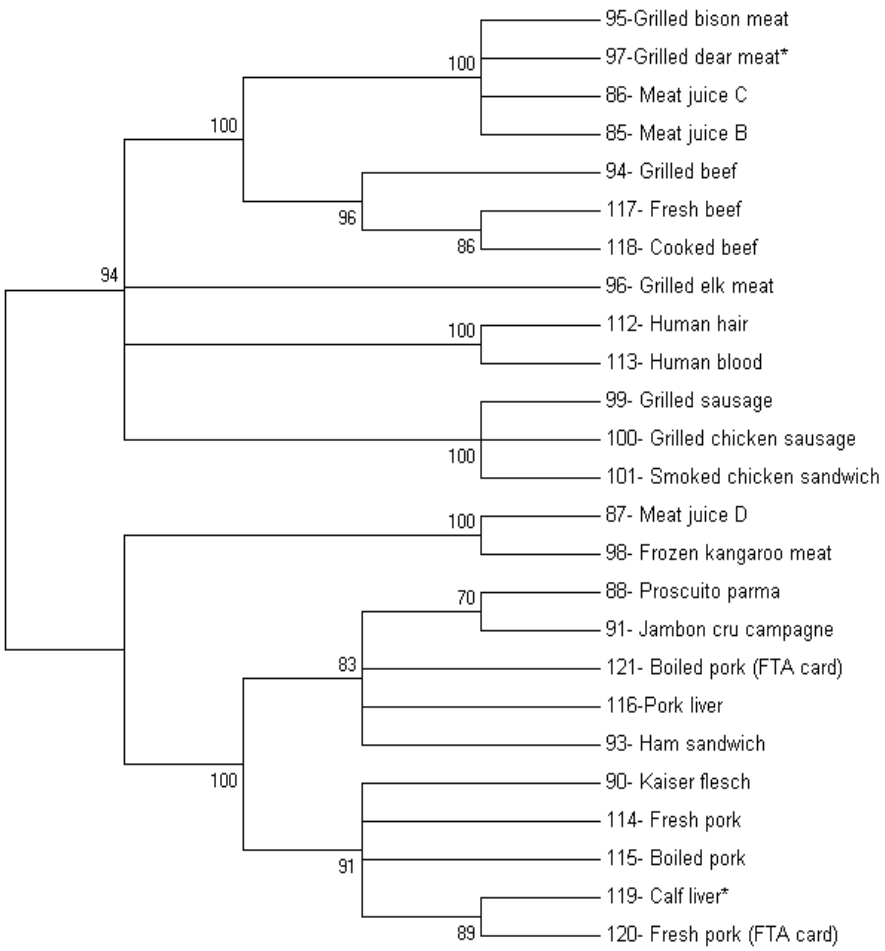


Figure 3.21: The phylogenetic tree of field specimens and their bootstrap values

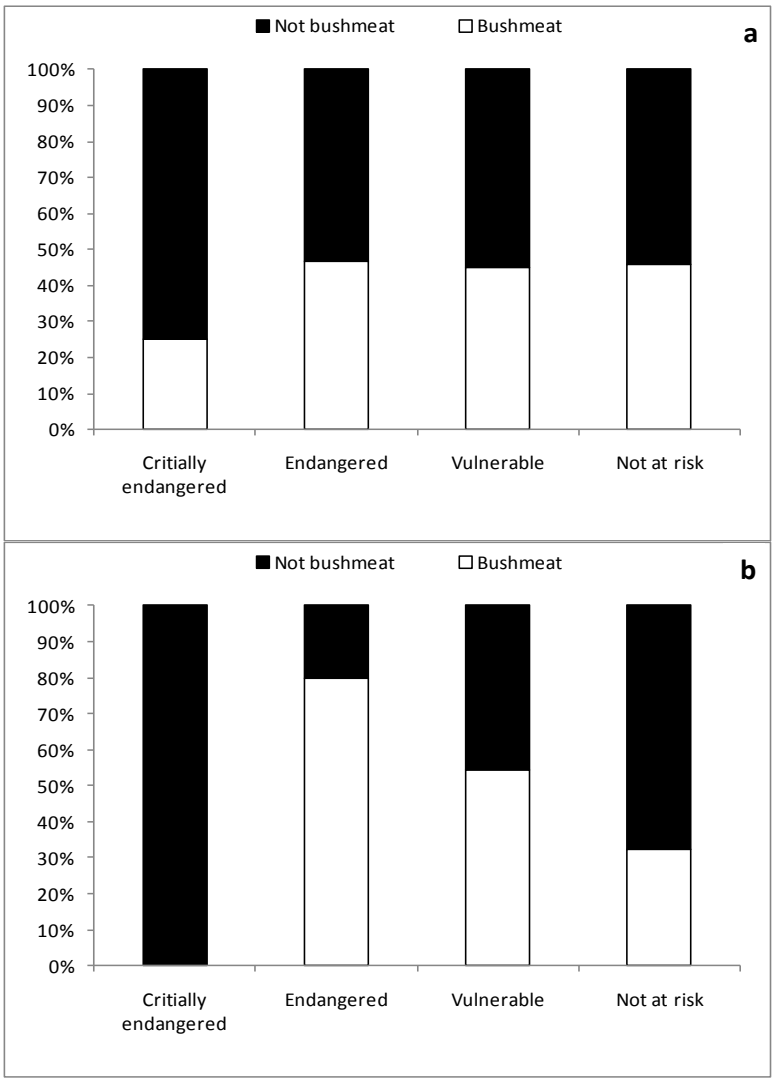


Figure 4.1: The percentage of threatened mammals hunted for bushmeat or not in relation to their status in Cameroon (a) and Ghana (b)