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Species Identification of Bushmeat Marketed by Using DNA Barcoding in the Region of Kisangani, D. R. Congo

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ABSTRACT

The Congo Basin Rainforest is characterized by a rich diversity in mammals. Scientific research demonstrated that this biodiversity is currently under high pressure. The survival of the fauna is threatened due to overexploitation as the demand for bushmeat as the primary source of animal protein increases due to the growing human population. This phenomenon is observed in the region of Kisangani, the biggest city in the northeast of the Democratic Republic of Congo (DRC).

Method: This study reports on the species diversity involved in the bushmeat trade in the region of Kisangani by means of DNA barcoding of mitochondrial DNA fragments cytochrome c oxidase subunit I (COI) and cytochrome b. An identification success rate of 65% was achieved (231 of 353 samples).

Research showed that in fact all mammal species are hunted, including endangered species as the chimpanzee (*Pan troglodytes)* and the okapi (*Okapia johnstoni).* Duikers (*Cephalophinae*) are the most important group of bushmeat species in terms of numbers (34%). Mammal species, except for rodents, become depleted in the close surroundings of Kisangani due to overexploitation. Mainly big-sized and medium-sized animals, which are profitable enough to compensate for the transport costs, are available on the market in Kisangani. Animals larger than 40 à 45 centimeters (shoulder height) are killed with shotguns, smaller animals are trapped. The bushmeat trade is explored more into detail by interviewing all parties involved in the bushmeat trade from hunters over sellers to the consumers, with regard to conservation of the fauna.

This dissertation proved overexploitation of wild animals in the region of Kisangani, DRC. Bushmeat consumption is reduced most effectively when it is becoming more expensive than alternative protein resources, rather than increasing the awareness on overexploitation or diseases emerging from wild animal consumption.

Keywords: Bushmeat trade, overexploitation, DNA barcoding, wildlife conservation, Kisangani region

INTRODUCTION

Studies have previously been performed in South-Africa (D'Amato et al. 2013), Tanzania (Bitanyi et al. 2011), Guinea-Bissau (Minhós et al. 2013), Cameroon and Nigeria (Fa et al. 2006) and Democratic Republic of the Congo and Gabon (Wilkie and Carpenter 1999) and other African regions. All these investigations are completed only very recently and the bushmeat trade in Africa is still understudied. The purpose of this report is to reconstruct the

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bushmeat trade in the Democratic Republic of Congo, more specifically in the region around Kisangani. It is a city counting approximately one million inhabitants and with a booming economy. All parameters for bushmeat overexploitation are present.

Previous studies on the diversity involved in bushmeat trade were based on morphological identifications by hunters, sellers or researchers themselves. Recent studies often apply DNA barcoding, which is a more reliable technique to identify smoke/burned bushmeat (Eaton et al. 2010).

The purpose of this study was to inventarize the bushmeat diversity involved in the bushmeat trade on the basis of molecular DNA analysis by barcoding. We hypothesize that species registered as 'Near Threatened' or 'Endangered' on the IUCN Red List are involved in bushmeat trade. Based on the molecular identifications, the research purpose is to investigate trends at different levels of the bushmeat trade. We want to evaluate if species are systematically sold under a different species name by comparing seller identifications with molecular identifications. Market data can be used to understand the taxonomic units to which exploited species belong and to gather data that allow for phyletic links at the level of various species exploited as bushmeat

The mammal diversity in this region is relatively well-known, but new species are still discovered. The lesula, a new species of *Cercopithecus* monkey, is described only two years ago (Hart et al. 2012). The most abundant mammal species occurring in central Africa are likely to be involved in the bushmeat trade (Petrozzi et al. 2016). Ituri-Aru (12.380 km²), Ituri-Epulu (15.720 km²), and Maiko (18.310 km²) are three high priority sites for conservation of biodiversity close to Kisangani with respectively the Okapi Faunal Reserve, Okapi Wildlife Reserve and Maiko National park (Figure 1).

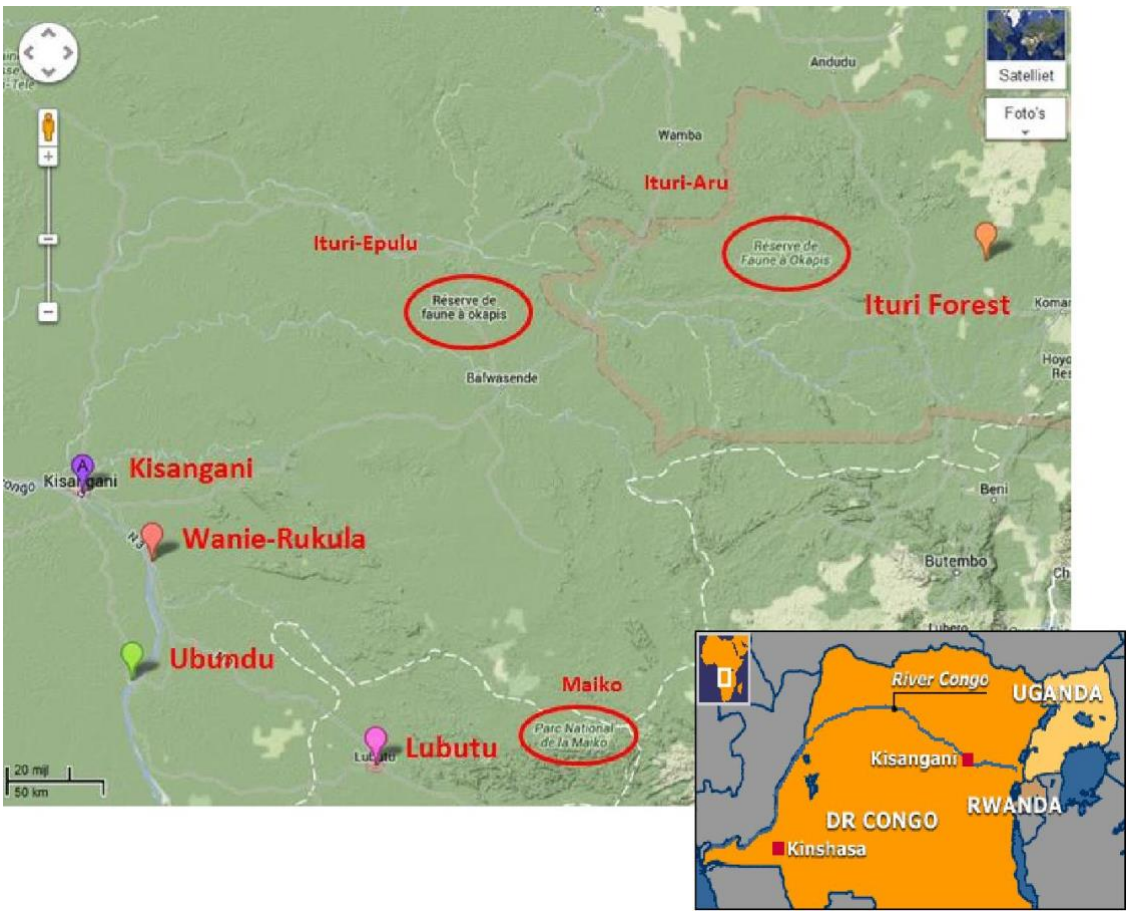

Figure 1: Geographical representation of sample sites

The Ituri Forest houses also the biggest variety of primates recorded in Africa (Hart et al., 1986). Populations of thirteen primate species, mainly Cercopithecidae, including the rare chimpanzee (*Pan troglodytes*), owl-faced monkey (*Cercopithecus hamlyni*) and l'Hoest's monkey (*Cercopithecus l'hoesti*) are established in the Ituri Forest (Oates 1996). Also the world's biggest population of Okapi's is found in the Ituri forest. Okapi's are together with the extremely rare fishing genet (*Osbornictis piscivora*) in the north east endemic to DRC (Sayer et al. 1992). The eastern lowland gorilla (*Gorilla berengei graueri*) can be found inside the Maiko National Park.

MATERIAL AND METHODS

Field Work: Data Collection

We collected samples at the central Market in Kisangani during the large central market and a small market across the Congo River in Kisangani (Isomela market). We obtained prior permission from market authorities and the informed consent from the traders (all of whom are women) to collect data on the trade of bushmeat. The data collection focused on regular traders present on a daily basis and with whom we had established a relationship of trust. On each visit all traders agreed to provide data on species, number, piece (entire animal, in pieces) and origin of the carcasses sold that day. We went on excursions in the direction of Banalia (128 Km), Sukisa (265 Km), Buta (324 Km), Ubundu (60 Km), Lubutu (85 KM) and Ituri (122 Km) (Figure 1). These directions were chosen because the highest bushmeat exploitation is expected in the region of Ituri near the Okapi Wildlife Reserve, in the region of Lubutu near the Maiko National Park, in the region of Buta near Rubi-Tele and Bili_Uere Park and in the region of Ubundu across the Congo River (natural barrier).

In total, 353 samples have been collected for molecular identification (202 inside Kisangani, 151 outside Kisangani).

Laboratory work is executed at the Royal Belgium Institute of Natural Sciences. The sampling procedure was performed as random as possible to obtain a representative database by visiting the markets in Kisangani at different days of the month (2 in the beginning, 2 in the middle and 2 at the end of the month), by visiting different market stalls and buying the different bushmeat species offered per stall. As such, the offered bushmeat diversity as well as the abundance per bushmeat species (common species are offered at more stalls than rare species) should be represented in the data collection.

The bushmeat tissues gathered throughout the day are wrapped in sacks. Writing down corresponding information about the sample on the sacks packing with a waterproof marker approved to be a very efficient working method. The samples are always put into collection tubes filled with 70% ethanol solution in the evening of the day there are collected. Tools used for the dissection are a scalpel, tweezers and scissors. These tools are disinfected with 70% ethanol and lighter in between each sampling procedure.

While gathering tissue samples of bushmeat. We consistently note four data down: (i) the place of collection, (ii) the direction (region) of origin, (iii) the hunting method and (iv) the morphological identification of the bushmeat species performed by sellers. In order to avoid too complex geographical analyses, the collection locations were only distinct as to be inside or outside Kisangani. Also, bushmeat sold at Kisangani was considered to originate from either direction/region Wanie-Rukula, Ubundu, Lubutu or Ituri that are respectively situated at 58 km, 150 km, 244 km and approximately 326 km from Kisangani. Guns or traps were the only hunting techniques applied. The morphological identification performed by sellers is compared with barcoding results.

Laboratory Work: DNA Barcoding

It is difficult to identify the species morphologically at the markets because they are mostly smoked or burned to extend the conservation period (Hajibabaei et al., 2006). Fresh meat would have been rotten before reaching the market. And they are often cut into pieces for the ease of trade. For this reason, the DNA barcoding technology (Hebert et al., 2003a; Hebert et al., 2003b) is used in order to identify the species sold on markets. This technique uses the cytochrome oxidase I (COI) or cytochrome b fragment embedded in the mitochondrial DNA of animals. COI and cytochrome b sequences mutate fast enough to separate closely related species and slow enough to be a reliable barcode for individuals of the same species (Brownlee 2004, Ivanova et al., 2007). The gathering of sequences in a database provides an overview of the species diversity involved in the bushmeat trade and helps to fight the trade of endangered species (Ratnasingham and Hebert 2007).

a) DNA extraction step

The DNA was extracted from the tissue samples using the Nucleospin® Tissue kit and following the included user manual provided by Macherey-Nagel (2012). Small pieces of approximately 25 mg from the collection tubes were transferred into numbered microcentrifuge tubes (1.5 mL). The protocol was run with 24 or 48 samples at the time. 180 µL of buffer T1 and 25 µL proteinase K solution was added in each microcentrifuge tube. It is recommended to prepare a mixture of 600 µL proteinase K and 4320 µL T1 buffer, vortex the mixture and then divide it over the 24 microcentrifuge tubes. The T1 buffer and proteinase K pre-lysed the samples while they were incubated overnight at 56 °C and 650 rpm. The samples were vortexed after the incubation. 200 µL buffer B3 was added to lyse the samples. The tubes were vortexed, before placing them back in the incubator at 70°C for 10 minutes. It is advisable to vortex again to make sure all tissue particles are dissolved well. Adding 210 µL 100% ethanol adjusted DNA binding conditions. The next step included a transfer of the mixture with the dissolved tissue samples into Nucleospin® Tissue Columns which were placed in collection tubes. The samples were vortexed 1 minute at 11.000 rpm.

The DNA binds on the matrix of the Nucleospin® Tissue Columns, while the fluid passes completely through the matrix and is captured by the collection tubes. The flow-through was discarded and the Nucleospin® Tissue Columns were placed back into the collection tubes. Afterwards, the silica membrane of the Nucleospin® Tissue Columns needed to be washed twice in order to purify the retained DNA. 500 µL BW buffer was used for the first wash. The samples were vortexed 1 minute at 11.000 rpm. The flow-through could be discarded. 600 µL B5 buffer was added for the second wash and the samples were centrifuged 1 minute at 11.000 rpm. The flow-through was discarded and samples were centrifuged again 1 minute at 11.000 rpm to dry the silica membrane of the columns. The tubes need to sweat out for a while to make sure all ethanol is removed. The complete removal is important because ethanol condenses the DNA and complicates the elution. Finally, the DNA had to be eluted from the matrix with 100 µL BE buffer (5mM Tris/HCl, pH 8.5) that must be preheated at 70 °C. The Nucleospin® Tissue Columns were placed in clean, labeled collection tubes and centrifuged 1 minute at 11.000 rpm. The flow-through was not discarded this time as it contained the extracted DNA. The extracted DNA per sample was pipetted into strips (8 tubes) or a plate (96 tubes) which can be covered with caps to store the samples in the freezer.

b) Control step with Nanodrop™ 1000 photospectrometer

The quality and quantity of extracted DNA was examined with the Nanodrop™ 1000 photospectrometer (Thermo Fisher Scientific). The nucleic acids program shows the measurement of the quantity expressed in ng per µL. The quality is represented as the λ260/ λ280-ratio. The photospectrometer was cleaned with a piece of paper after each measurement to obtain reliable results. The first measurement needs to be performed with pure water, then the device has to be calibrated with buffer BE, functioning as blanco measurement. Ideally the

DNA quantity of the extractions should be around 50 ng/µL and the DNA quality 1.8.

c) Polymerase Chain Reaction step

Molecular identification is commonly based on either the cytochrome oxidase I (COI) fragment or the cytochrome b fragment (Hebert et al., 2003a; Hebert et al., 2003b). These DNA fragments produce enzymes that play an essential role in the metabolism of all aerobe organisms, the sequences can be copied applying special designed primers. Following primers were used for the amplification of COI fragments: VF1_t1, VR1_t1, VF1d_t1, VR1d_t1 (Ivanova et al. ,2006, Appendix 1). The 'V' stands for vertebrates, the 'F' for forward primer, the 'R' for reversed primer.

An extra pair of primers, specifically for Lepidoptera, was applied, namely LepF1_t1 and LepR1_t1 (Eaton et al., 2010, Appendix 1). The primers for Lepidoptera (butterflies) are much shorter than the ones specifically for vertebrates. These mini-barcodes can be used for a greater success in the amplification of degraded DNA extracted from burned mammal tissues (Hajibabaei et al., 2006). Stock primer solutions have a concentration of 100 µM and need to be diluted into a working solution. The primer concentration in the working solution called 'primer cocktail' is 2µM. In order to achieve this concentration, 12 µL primer solution (2 µL of each primer) was diluted with 88 µL H_2O. The primer cocktail was vortexed and stored in the freezer.

The preparation of the primers for amplification of cytochrome b fragments is, except for the primers, the same. The cytochrome b primers used were: forward primer L14816 and reverse primer H15173 (Bravi et al. 2004; Appendix 1). The sequences (COI or cytochrome b) of species expected to be identified were checked beforehand, on their availability in GenBank or BOLD. (Appendix 2).

The primer cocktail is one of the components of the master mix for the polymerase chain reaction (PCR). Others are PCR-buffer, dNTPs, H_2O and Taq (Table 1). Deoxynucleotide triphosphate (dNTP) solution contains nucleotides (adenosine, cytosine, guanine and thymine) which are the construction units of DNA. Taq polymerase is a thermostable DNA polymerase. It is able to withstand the high temperature conditions during the PCR procedure and catalyze the binding of the primers during the denaturation cycle. A PCR was performed for each sample separately. A volume master mix was prepared in one tube corresponding to the amount of samples. The master mix was vortexed carefully to avoid enzyme damage. A little surplus was always prepared to make sure there was no deficit when the master mix was divided into clean, labelled tubes. 23 µL was pipetted in each tube. Afterwards, 2 µL of the solution with extracted DNA was pipeted in the tubes. It was pipetted up and down to mix the DNA with the master mix.

The tubes containing master mix were placed in the PCR block (PCR Thermocycler Biometra Tpersonal) for amplification of the COI or cytochrome b fragments. The PCR-cycle consist of three processes: denaturation, annealing and elongation (Eaton et al. 2010; Appendix 3). The denaturation of both DNA-strings is necessary for the primers to attach. The DNA-strings are driven apart at 94 °C. The attachment of primers on the matching DNA sites is called annealing and occurs at 52 °C. At this moment the Taq polymerase binds to the primer template and starts copying the COI or cytochrome b fragment.

New DNA is constructed with dNTP's. The elongation step takes place at 72 °C. The cycle (phase 2, 3 and 4 of the temperature scheme) is repeated 40 times for exponential amplification of the fragments. After the PCR, huge concentration of the targeted DNA fragment occurs in the solution.

Table 1: Amounts of reagents needed per sample amplification and temperature scheme

COI			
	Master mix (µL)		**Temperature scheme (°C)**
PCR-buffer (10x)	2.5	120 sec	94
dNTP	2.5	30 sec	94
Primer cocktail (2 µM)	2.5	40 sec	52
H_2O	15.5	60 sec	72
Taq	0.2	600 sec	72
DNA	2	300 sec	5
		Pause	15
CYT B			
	Master mix (µL)		**Temperature scheme (°C)**
PCR-buffer (10x)	2.5	90 sec	94
dNTP	2.5	30 sec	94
primer L14816 (2 µM)	5	30 sec	50
primer H15173 (2 µM)	5	45 sec	72
H_2O	8	600 sec	72
Taq	0.2	300 sec	5
DNA	2	Pause	15

d) Control step with gel electrophoresis

The next step implied expensive solutions, so spilling had to be avoided. Failed PCR-products were removed out of the analysis by a gel electrophoresis. Following process steps were done: prepare a gel with 1.2% agarose fluid. Place combs in the holder to provide loading points. Let the gel harden for 15 minutes. In the meantime, stain 3.5 µL DNA of each sample with 1.5 µL Coomassie Brilliant Blue liquid. Pipet the stained DNA-solutions and a DNA-ladder (Fermentas: GenRulerTM 100bp plus DNA Ladder # SM0321) when the agarose gel is ready. Run the gel electrophoresis for 37 minutes at 100 V. Put the gel subsequently in a bath of GELRED. Staining with GELRED makes it possible to see how far the segments moved across the gel under UV-light. Shorter fragments experience less resistance and will travel further into the gel. COI is amplified if the DNA-fragments are at the height of approximately 657 base pairs marked by the ladder. The DNA fragments must be at the height of the approximately 375 base pairs on the ladder if cytochrome b was extracted.

e) Purification step through ultrafiltration with Nucleofast®

Two bands can be detected on the image of the gel electrophoresis. One band contains the DNA-fragments and the other band contains the residual primers. The primer fragments are considerably smaller than the DNA fragments and have travelled the longest distance through the gel. As the sequence of the gene is the interesting part, for this reason the primers need to be removed from the solution. This was done with Nucleofast® which is a simple procedure whereby special filters separate the short primers from the long DNA-fragments (figure 2). The PCR products were loaded in a Nucleofast® filter plate and a vacuum under-pressure of 500 mbar was created to suck the solution through the filters. After 15 minutes, 30 µL nuclease-free water was added to recuperate the purified DNA-fragments on top of the filters. It is recommended to wait at least 5 minutes and pipet up and down several times to get the condensed DNA back into solution, before pipetting the solution into clean strips.

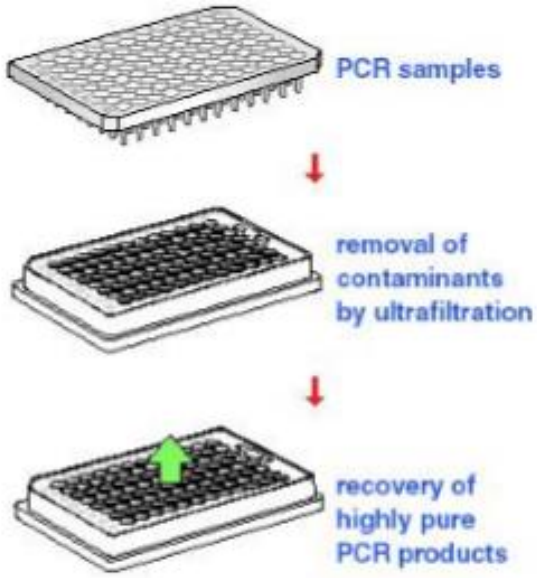

Figure 2: Purification through ultrafiltration with Nucleofast

f) DNA sequencing

The order of the nucleotides in DNA fragments was determined with DNA sequencing whereby the fragments are separated by denaturation and primers initiate the copying of each strain. NTPs are available for the elongation process. Next to NTP's, there are also ddNTPs present. The elongation process stops when dideoxynucleotides (ddNTPs) are implemented because they do not contain a 3'-hydroxyl group which is necessary to bind two nucleotides (Appendix 5). ddATP, ddTTP, ddGTP, ddCTP are respectively labelled with a green, red, black and blue fluorescent dye to make them visible for the ABI Prism 3130 xl capillary sequencer later in the process. The protocol is executed for forward and reverse sequences separately (see PCR step for primers). Two master mixes are prepared (Table 2). BigDye® Terminator v3.1 Ready Mix is light sensitive and solutions containing it should be kept in a dark environment as much as possible. The forward and reverse master mix was vortexed and 8µL was divided in the tubes of respectively a forward and reverse plate with 96 tubes. 2 µL DNA-product, obtained from the Nucleofast® step, for each sample was added in the tubes. The DNA-products were pipetted up and down into the tubes to mix. Then, the plate covered with strips was placed in the PCR-block (Thermal Cycler Biometra T3000).

After the PCR, a precipitation step was performed to purify the DNA-fragments and remove the unincorporated dye terminators because they would difficult the analysis of the sequence in the ABI Prism 3130xl Genetic Analyzer. 5 µL ethylenediaminetetraacetic acid (EDTA, 0.125 M) and 60 µL freezing cold ethanol (EtOH, 100%) was added in each tube. The plate was subsequently covered with caps and then carefully vortexed before putting it in the freezer for 15 minutes. The EDTA, ethanol and cold temperatures enhance the precipitation of DNA at the bottom of the tubes. The PCR-products in contrast stay dissolved to be separated from DNA. The DNA was further precipitated by centrifuging the solution 45 minutes at 4000 rpm. The DNA sticks now to the bottom of the plate. The superfluous liquid with redundant PCR-products can be removed by turning the plate upside down on a tissue and vortex it a few seconds at 200 rpm. The process was repeated with 70 µL fridge-cold 70% and 15 minutes of centrifugation. The plate has to rest during 30 minutes under a box for total evaporation of the remaining ethanol. Ethanol has to disappear completely because it has a condensing effect on

DNA which complicates the sequence analysis. The DNA was put back into solution with 15 µL toxic Super-DI™ Formamide. Formamide will denature the DNA-strings. The sequence analyzer requires single stranded DNA-sequences to analyze. The plate was also heated for a few minutes at 95 °C in the PCR-block to promote denaturation. Lastly, the plate was cooled to 5°C in the PCR-block before placing it into the sequence analyser for the identification of the samples.

Table 2: DNA sequence master mix preparation and PCR-block temperature scheme for COI and Cyt

COI / CYT B			
	Master mix (µL)		Temperature scheme (°C)
PCR-buffer (5x)	1.5	240 sec	96
BigDye® Terminator v3.1 Ready Mix	1	10 sec	96
Forward OR Reverse primers (2 µM)	1.5	5 sec	50
H_2O	4	240 sec	60
PCR-DNA	2	pause	4

Fragments migrate through the electric fields of the sequence in the order of their size (ddNTPs are implemented at different moments; Figure 3). A detector displays the fluorescent signals as peaks over time in an electropherogram. The order of the fluorescent peaks corresponds to the nucleotide sequence.

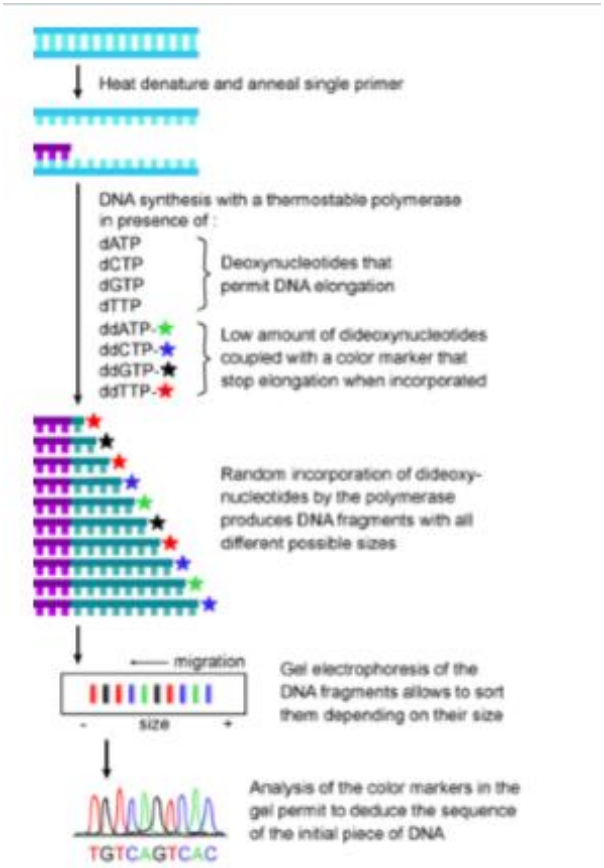

Figure 3: Schematic overview of DNA sequencing process

The electropherograms of the forward and reverse sequences are assembled with the program CodonCode Aligner to create a consensus sequence. CodonCode Aligner allows to

correct nucleotide mistakes as a consequence of mismatches between the peaks of the forward and reserse electropherograms. Samples with bad quality forward and/or reverse sequences are discarded and put through the DNA sequencing process over again. The corrected good quality sequences are aligned in MEGA5.2 and prepared for Nucleotide BLAST (Basic Local Alignment Search Tool) in the database of Genbank or BOLD on the internet. These public databases contain sequences of already identified species which can be used to identify my samples. This is a phylogenetic approach based on the fact that interspecies divergence is higher than intraspecies divergence. Species identity is used as simple measure of evolutionary divergence, with rules of thumb like >97% indicates the same species and >95% the same genus (BLAST manual). The divergences are visualized by constructing a phylogenetic tree for the COI and cytochrome b sequences (Statistical Method: Neighbor-Joining Tree; Test of Phylogeny: Bootstrap 1000 replications; Model: p-distance; Included Nucleotide Substitutions: Transitions and Transversions; Gaps: pairwise deletion). The topology of the phylogenetic tree is considered correct for bootstrap values > 95% (Nei and Kumar 2000; Appendix 6, 7 and 8).

The molecular identifications, instead of unreliable morphological identifications performed by sellers or hunters, are used to investigate trends in bushmeat trade. Analyses of trends are performed in Excel (see fieldwork: data collected by interviewing locals). Statistical evidence is calculated using program R3.1.1. I applied the Chi-squared Test of Independence (De Merode et al. 2004) to check the significance of trends.

Test Protocol

Three tests were performed to check whether the species identification success rate could be improved. For each test 2 samples were selected that did work on COI, 2 other samples that did not work on COI, but work on cytochrome b, 3 samples that do not work on COI nor cytochrome and 1 Blanco sample as control. A PCR master mix for 9 samples was prepared per test (Table 3). The difference in amplification success (checked with gel electrophoresis) between the different tests will show which master mix is optimal.

Table 3: Test protocols for optimization of identification success rate

Test 1: Different concentrations $MgCl_2$					
	Master mix for 1 sample (µL)				**Temperature scheme (°C)**
	1.5 mM	**2.5 mM**	**3.5 mM**		
PCR-buffer (10x, RED)	2.5	2.5	2.5	120 sec	94
dNTP (2 mM)	2.5	2.5	2.5	30 sec	94
Primer cocktail (2 µM)	2.5	2.5	2.5	40 sec	52
Taq (5 U/µL)	0.2	0.2	0.2	60 sec	72 (40 cycli)
$MgCl_2$	0	1	2	600 sec	72
H_2O	15.5	14.5	13.5	300 sec	5
DNA	2	2	2	Pause	15
Test 2: Different primer concentrations					
	Master mix for 1 sample (µL)				**Temperature scheme (°C)**
	0.1 mM	**0.2 mM**	**0.4 mM**		
PCR-buffer (10x, RED)	2.5	2.5	2.5	120 sec	94
dNTP (2 mM)	2.5	2.5	2.5	30 sec	94
Primer cocktail (2 µM)	1.25	2.5	5	40 sec	52
Taq (5 U/µL)	0.2	0.2	0.2	60 sec	72 (40 cycli)
$MgCl_2$	0	0	0	600 sec	72
H_2O	16.75	14.5	13	300 sec	5
DNA	2	2	2	Pause	15
Test 3: With or without using multiplex PCR-kit (Qiagen)					
	Master mix for 1 sample (µL)				**Temperature scheme (°C)**
	With Q		**Without Q**		
Qiagen (2x)	5		5	900 sec	95
Q solution	1		1	30 sec	94
Primer cocktail (2 µM)	0		1	90 sec	52
H_2O	2		1	60 sec	72 (40 cycli)
DNA	2		2	600 sec	72
				300 sec	5
				Pause	15

RESULTS

Identification Success Rate

Some hurdles were faced during the molecular identification process. The measurements with NanodropTM photospectrometer revealed sometimes strange values (Table 4). The DNA-quantity and DNA-quality did mostly deviate considerably from optimal values 50 ng/µL and 1.8 respectively. However, gel electrophoresis proved that this was not a problem for amplification of DNA-fragments with PCR. A sufficient high concentration of DNA-fragments was obtained for most samples. The molecular identification procedure was completed for 353 samples and DNA-sequences were achieved for 231 of them. This resulted in a success rate of 65%. The reason for failed sample identifications might be the very poor DNA quality of those samples as a consequence of burning the meat for conservation. The procedures of the DNA barcoding protocol did not have an influence. The identification success rate could not be improved by any of the three test protocols.

Diversity Involved in Bushmeat Trade

The total diversity of collected species by using COI and Cyt b is represented in Table 5, together with their IUCN status and D.R.Congo protection status, and how many times they were collected (phylogenetic trees: Appendix 6 and 7).

Table 4: List of species collected in the region of Kisangani and their IUCN Red List status

No	Scientific name	English name	IUCN Red List		Collected	
			Status	Trend	Nr.	%
1	*Aonyx capensis*	African clawless otter	LC	Stable	1	0.43
2	*Atherurus africanus*	African brush-tailed porcupine	LC	Unknown	5	2.17
3	*Atilax paludinosus*	Marsh mongoose	LC	Decreasing	1	0.43
4	*Cephalophus callipygus*	Peter's duiker	LC	Decreasing	8	3.48
5	*Cephalophus dorsalis*	Bay duiker	LC	Decreasing	55	23.54
6	*Cephalophus monticola*	Blue duiker	LC	Stable	11	4.78
7	*Cephalophus nigrifrons*	Black-fronted duiker	LC	Decreasing	1	0.43
8	*Cephalophus rufilatus*	Red-flanked duiker	LC	Decreasing	1	0.43
9	*Cephalophus silvicultor*	Yellow-backed duiker	LC	Decreasing	10	4.35
10	*Cercocebus chrysogaster*	Golden-bellied mangabey	DD	Decreasing	1	0.43
11	*Cercopithecus ascanius*	Red-tailed monkey	LC	Unknown	12	5.22
12	*Cercopithecus denti*	Dent's mona monkey	LC	Uknown	2	0.87
13	*Cercopithecus hamlyni*	Owl-faced monkey	VU	Decreasing	2	0.87
14	*Cercopithecus kandti*	Golden monkey	EN	Unknown	4	1.74
15	*Cercopithecus l'hoesti*	L'hoest's monkey	VU	Decreasing	1	0.43
16	*Colobus angolensis*	Angola colobus	LC	Unknown	1	0.43
17	*Colobus badius*	Western red colobus	EN	Decreasing	2	0.87
18	*Cricetomys emini*	Emin's pouched rat	LC	Stable	20	8.70
19	*Heliosciurus gambianus*	Gambian sun squirrel	LC	Unknown	1	0.43
20	*Hyemoschus aquaticus*	Water chevrotain	LC	Decreasing	2	0.87
21	*Malacomys longipes*	Big-eared swamp rat	LC	Unknown	1	0.43
22	*Okapia johnstoni*	Okapi	EN	Decreasing	5	2.17
23	*Orycteropus afer*	Aardvark	LC	Unknown	3	1.30
24	*Pan troglodytes*	Chimpanzee	EN	Decreasing	13	5.65
25	*Panthera pardus*	Leopard	NT	Decreasing	1	0.43

26	*Papio anubis*	Olive baboon	LC	Increasing	2	0.87
27	*Petrodromus tetradactylus*	Four-toed elephant shrew	LC	Stable	2	0.87
28	*Poiana richardsonii*	African linsang	LC	Unknown	1	0.43
29	*Potamochoerus porcus*	Red river hog	LC	Decreasing	22	9.56
30	*Potamogale velox*	Giant otter shrew	LC	Decreasing	1	0.43
31	*Tragelaphus spekii*	Sitatunga	LC	Decreasing	7	3.04
Identification of 'no hit' COI and cytochrome b sequences to (sub)family level						
33	Cercopithecidae	Old world monkeys	/	/	2	0.87
37	Galagidae	Galagos	/	/	13	5.65
32	Lutrinae	Otters	/	/	2	0.87
34	Manidae	Pangolins	/	/	2	0.87
35	Murinae	Old world rats and mice	/	/	5	2.17
36	Protoxerini	African squirrels	/	/	8	3.48

In the Table 4, we represented the data collection locations of species identified. There are 32 samples of which the sequences were defined, but no matching sequence could be found in GenBank or BOLD with 95% or more identical base pairs. These 32 samples, counting for 15% of the successfully analysed samples, are called 'no hits' (Table 4). It is unlikely though that a new species was discovered since these databases do not contain the sequences of all so far discovered species. It was possible though to define to which (sub) family the 'no hit'-species belongs to with phylogenetic trees (Appendix 8). The intraspecific differences shown by phylogenetic trees could be linked to geographic distribution, which is referred to as phylogeography (Stanton et al., 2014).

Systematic Identification Mistakes Made by Sellers

The identification of each sample performed by sellers is compared with the corresponding molecular identification achieved by barcoding and it is considered as correct, almost correct or incorrect (Figure 4). 118 out of 197 seller identifications (60%) appeared to be correct. Another 37 samples (19%) are determined almost correct. Almost correct means that the identification performed by sellers and the molecular identification turns out to be the same species, but the mistake is made on a subspecies level. Most determination mistakes made at subspecies level involved duiker species (Cephalopinae). This was the case for 36 of the 37 samples. Once a tissue was identified as *Cercopithecus ascanius*, but turned out to be *Cercopithecus denti*. Finally, 43 of the samples (21%) appeared to be identified incorrectly by sellers. The incorrect identifications are listed (Table 6) to discover systematic mistakes made by sellers.

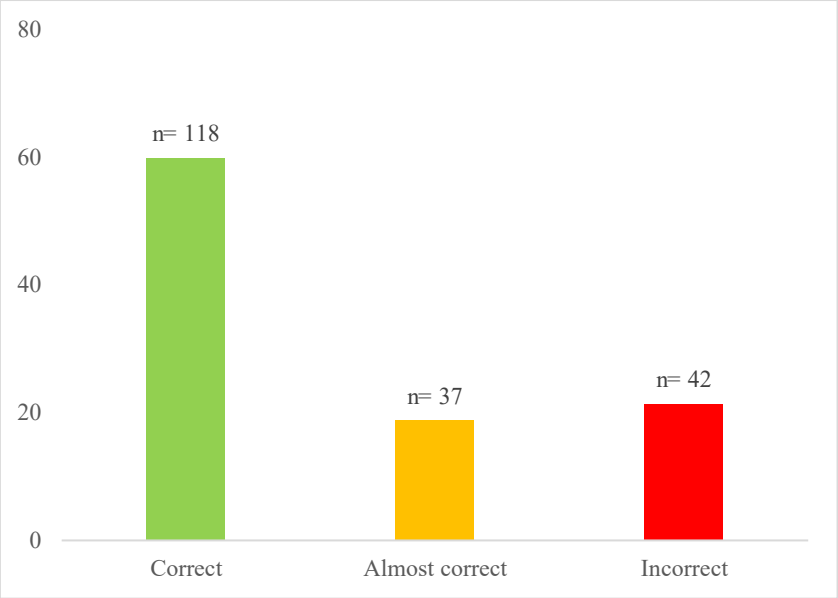

Figure 4: Comparison of morphological (sellers/hunters) with molecular (barcoding) identification

Table 5: Incorrect identifications by sellers

Seller identification (#)	Molecular identification (#)
Syncerus caffer nanus (9)	*Okapia johnstoni (4), Cephalophus silvicultor (3), Tragelaphus spekii (2)*
Potamochoerus porcus (6)	*Orycteropus afer (1), Cephalophus dorsalis (3), Pan troglodytes (1), Cercopithecus ascanius (1)*
Hyemoschus aquaticus (3)	*Cephalophus dorsalis (1), Cephalophus silvicultor (2)*
Cephalophus silvicultor (1)	*Potamochoerus porcus (1)*
Cephalophus dorsalis (3)	*Potamochoerus porcus (2), Cercopithecus ascanius (1)*
Cephalophus nigrifrons (2)	*Tragelaphus spekii (2)*
Pan troglodytes (2)	*Colobus badius (1), Cercopithecus ascanius (1)*
Okapia johnstoni (1)	*Cephalophus dorsalis (1)*
Tragelaphus spekii (3)	*Cephalophus silvicultor (3)*
Cercopithecus sp. (5)	*Tragelaphus spekii (1), Cephalophus callipygus (1), Cephalophus dorsalis (2), Colobus badius (1)*
Atilax paludinosis (1)	*Tragelaphus spekii (1)*
Papio (1)	*Pan troglodytes (1)*
Civettis civetta (1)	*Aonyx capensis (1)*
Thryonomis swinderianus (2)	*Atherurus africanus (1), Heliosciurus gambianus (1)*
Crossarchus alexandri (1)	*Orycteropus afer (1)*
Atherurus africanus (1)	*Cricetomys emini (1)*

DISCUSSION

Diversity Involved in Bushmeat Trade

Hunters yearly extract 6 million tonnes of animals from the rainforest worldwide (Nasi et al., 2011). The results confirm that a wide diversity of mammals are chased (Table 4). The diversity of the collected bushmeat samples is to such an extent that it can be assumed all mammal species are being hunted in the region of Kisangani. All kinds of mammals are targeted: small (Rodents) or large (Ungulates), arboreal (Cercopithecidae) or non-arboreal (Cephalophinae), carnivora (*Panthera pardus*) or herbivores (*Okapia johnstoni*. Hunters and sellers also do not take into account the species status on the IUCN Red List. The majority of

mammal species (70%) hunted in the Congo Basin is not listed as threatened on the IUCN Red List of Threatened Species (Nasi et al., 2011), in Kisangani only 19% of the hunted species is listed as threatened. A high number of killed Cephalophinae, Cercopithecidae, *Pan troglodytes*, *Potamochoerus porcus* and *Cricetomys emini* were collected. Duikers account for 34% of the collected bushmeat samples, which is in line with previous research stating that duikers are the most important bushmeat species in terms of biomass (Nasi et al., 2011). A major concern is the high number of kills observed for primate species as several of them are reported to be vulnerable or endangered by the IUCN Red List. Hunters apparently seem not to make an effort to distinguish rare species from their more abundant relatives. Both Cercopithecidae (*Cercopithecus ascanius* and *Cercopithecus denti*) registered as Least Concern on IUCN Red List and Cercopithecidae (*Cercopithecus hamlyni* and *Cercopithecus l'hoesti*) registered as 'Vulnerable' are hunted. Also a considerable number of *Okapia johnstoni* carcasses is found on the markets, although it is endangered and protected by law as the national symbol for nature conservation in DRC. The *Panthera pardus* holds a vital ecological role as the sole large predator in the rainforests of the Congo Basin (Scholes et al., 2006). The discovery that also this mammal is involved in bushmeat trade is far from reassuring regarding the preservation of the ecosystem in the region around Kisangani. Larger species tend to disappear first in the harvest as they are more vulnerable than smaller species to overharvesting (Robinson and Bennett, 2004).

Sellers

Bushmeat is often cut into small pieces at markets, so consumers cannot see which species they are buying and trust the sellers. However, one in five bushmeat pieces is not sold as the actual species. This might happen unintentionally due to lack of knowledge or unidentifiable state of bushmeat as it is smoked or burned for conservation (Margaret et al., 2003). But systematic mistakes suggest intentional deception. There are several conceivable reasons for sellers (and hunters) to delude consumers: (i) get rid of less tasty species, (ii) fetch higher prices for cheap bushmeat species and (iii) hide trade of illegal species. Detailed analysis revealed one systematic mistake being *Okapia johnstoni, Cephalophus silvicultor* or *Tragelaphus spekii* sold as *Syncerus* caffer (Table 7), but not one *Syncerus caffer* is actually available on the markets according the dataset (Table 6). This might be an artefact, but possibly *Syncerus caffer* disappeared from the bushmeat trade because it became extinct in the region of Kisangani. The reason for selling other bushmeat species as *Syncerus caffer* is not to get higher prices as it is was the cheapest bushmeat available. *Tragelaphus spekii* is not tasty as it is not even included in the list of most preferred species, while *Syncerus caffer* is ranked second after *Cephalophinae* (Dauwe, 2014). And the price for both bushmeat species is comparable, so the less tasty *Tragelaphus spekii* might be sold under the name of tasty *Syncerus caffer.* The same motive might explain the selling of *Tragelaphus spekii* or other species as *Cephalophinae* (Table 5). The difference in taste *between Syncerus caffer* and *Okapia johnstoni* is not remarkably (Dauwe, 2014) and *Okapia johnstoni* is much more expensive than *Syncerus caffer,* yet *Okapia johnstoni* is regularly sold as *Syncerus caffer* (Table 5). This could be an indication for intentional hiding of illegal bushmeat trade because most sellers are believed to be well aware of the protected status of the *Okapia johnstoni*.

CONCLUSIONS

The reconstruction of bushmeat trade in the region of Kisangani, DRC, revealed that all mammals are hunted, no matter their IUCN status. Wildelife are at great risk of disappearing in the region of Kisangani. Current legislation hunting and conservation is not respected, fraud persists (closing, opening hunting, and protected species). The overexploitation of big-sized

bushmeat species, like *Pan troglodytes* and *Okapia johnstoni*, is alarming because these slow reproducing animals cannot maintain healthy population sizes.

The responsibility for conservation of wild fauna lies with the government to provide financial support for the deployment of enough rangers to enforce (new) laws regarding bushmeat trade.

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